Complement Function and Expression in the Red-Tailed Phascogale (*Phascogale calura*)

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STATEMENT OF AUTHENTICATION

This thesis is submitted to the Western Sydney University in fulfilment of the requirement is for the Degree of Doctor of Philosophy.

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I, hereby declare, that I have not submitted this material, either in full or in part, for a degree at this or any other tertiary institution.

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3rd November 2016

PREFACE

This thesis is presented as a series of manuscripts, which describes the function and expression of the complement system in red-tailed phascogales (*Phascogale calura*). The work presented is either published, accepted for publication or is currently under review. An introductory and concluding literature review will be included in this thesis to assist with the continuity between manuscripts. References and formats of manuscripts will vary according to guidelines of journals that the manuscripts are submitted to. All manuscripts are jointly authored, but in each case, I am first author. An additional manuscript is attached to this thesis, showing additional work that I have done throughout my PhD.

PUBLICATIONS

- Stannard, HJ, Borthwick, CR, Ong, O, & Old, JM 2013, 'Longevity and breeding in captive red-tailed phascogales (*Phascogale calura*)', *.Australian Mammalogy,* vol. 35(2), pp. 217-219.
- *Ong, OTW, Young, LJ, & Old, JM 2015, 'Detection of an active complement system in red-tailed phascogales (*Phascogale calura*)', *Comparative Clinical Pathology*, vol. 24(6), pp. 1527-1534.
- *Ong, OTW, Young, LJ, & Old, JM 2016, 'Preliminary genomic survey and sequence analysis of the complement system in non-eutherian mammals' *Australian Mammalogy*, vol. 38(1), pp. 80-90.
- *Ong, OTW, Young, LJ, & Old, JM 2016, 'The expression of specific complement proteins in developing red-tailed phascogales (*Phascogale calura*)' *Developmental & Comparative Immunology*, vol. 65, pp. 314-320.
- *Ong, OTW, Young, LJ, & Old, JM 2016, 'Evaluation of housekeeping genes for gene expression in red-tailed phascogale liver, lung, small intestine and spleen' *PeerJ*, vol. 4, pp. e2552.
- *Ong, OTW, Green-Barber, JM, Kanuri, A, Young, LJ, & Old, JM 2016, 'Antimicrobial activity of red-tailed phascogale (*Phascogale calura*) serum' *Comparative Immunology, Microbiology and Infectious Diseases*, vol. 51, pp. 41-48.
- Green-Barber, JM, Ong, OTW, Kanuri, A, & Old, JM, 'Health and disease in free-ranging eastern grey kangaroos (*Macropus giganteus*)' Under Review in *Australian Mammalogy.*

***Manuscript used for this thesis**

CONFERENCE AND SEMINAR PRESENTATIONS

- Ong, OTW, Young, LJ & Old, JM. *The Presence of a Complement System in Red-tailed Phascogales (Phascogale calura)*. 2014. Presented at the North American Comparative Immunology, Albuquerque, New Mexico.
- Ong, OTW, Young, LJ & Old, JM. *The Identification of the Marsupial Complement System*. 2013. Presented at University of Western Sydney, Sydney, New South Wales.
- Ong, OTW. *Complement – Key to Marsupial Survival?* Presented for the 3 Minute Thesis (3MT) Competition, 2013 University of Western Sydney, Sydney, New South Wales.

ABSTRACT

The complement system is found in both invertebrates and vertebrates, and functions in both innate and adaptive immune responses. This thesis involves determining the presence of a complement system in non-eutherian mammals using publicly available non-eutherian genomes, followed by analysing the expression and effectiveness of the complement system in a model dasyurid, the red-tailed phascogale (*Phascogale calura*). Data mining was used to look for complement sequences in non-eutherian mammals, Polymerase Chain Reaction (PCR) to elucidate major complement component cDNA sequences, real-time PCR to determine the expression of those major complement components in developing phascogales, haemolytic assays to detect the presence of a functional complement system, and antimicrobial susceptibility testing to determine the antimicrobial capabilities of phascogale serum.

Use of online search tools and algorithms resulted in the successful identification of 27-47 complement and complement-related sequences in gray short-tailed opossum (*Monodelphis domestica*), tammar wallaby (*Macropus eugenii*), Tasmanian devil (*Sarcophilus harrisii*), koala (*Phascolarctos cinereus*) and platypus (*Ornithorhynchus anatinus*) genomes. Key complement proteins, specifically involved in the Classical, Alternative and Lectin Complement pathways, were also compared with homologous human protein sequences, which then allowed for the identification of functionally important motifs and conserved sequences. The identification of complement sequences in non-eutherian genomes possibly indicate a comparable complement system to eutherian mammals, therefore the expression and functionality of the complement system was investigated in a marsupial.

Real-time PCR (qPCR) has the ability to quantitate gene expression, indicating the presence of a functional gene product in a tissue. However experimental gene expression needs to be normalised against the expression of two stable reference genes. The stability of reference genes should have unregulated transcription in the various experimental conditions including differences in age, sex and tissues. Hence determining the stability of reference genes in different tissues and ages would be useful for marsupial gene expression studies. The expression of reference genes; Glyceraldehyde 3-Phosphate Dehydrogenase (*GAPDH*), Actin-β (*ACTB)*, 18S rRNA (*18S*), 28S rRNA (*28S*) and Ribosomal Protein L13a (*RPL13A*), were investigated in liver, lung, small intestine and spleen tissues of phascogales, and at least two stable reference genes were determined per tissue. The expression stability differed according to tissue sample and age, suggesting that the analysis of reference genes according to the type of tissue and ages needs to be considered in every gene expression study. Combining the expression in juveniles and adults, *GAPDH* was most stable in liver and lung tissues, and *18S* in small intestine and spleen tissues.

The relative expression of complement component 1, subcomponent r (C1r), complement component properdin (CFP), mannan-binding lectin serine peptidase 2 (MASP2) and complement component 3 (C3) were examined in pouch young, juvenile and adult phascogales using qPCR. The complement components were chosen because of their importance in the Classical, Alternative and Lectin Complement pathways. Average complement expression of all four complement components in whole body tissues showed a significant increase in developing pouch young. However complement expression decreased in adults compared to juveniles. All juvenile and adult phascogales were males, and may indicate a link between complement expression and male semelparity. Partial cDNA sequences for C3 and MASP2 were also extracted from phascogale liver tissue using RACE-PCR, and comparisons with respective eutherian homologues revealed the conservation of

functionally important sequences and motifs. This is the first time MASP2 has been suggested to be present and expressed in a marsupial, which indicates the presence of a functional Lectin Complement pathway in the phascogale.

Complement proteins have been found in mammalian serum. The effectiveness of phascogale complement serum was tested using standard haemolytic assays. The presence of a Classical and Alternative Complement pathway was confirmed, with female phascogale serum being more effective at lysing foreign erythrocytes, reinforcing the possible link between the complement system and semelparity. The results obtained indicate that the function of the Classical and Alternative Complement pathways in phascogales is comparable to other vertebrates.

Along with complement proteins, eutherian serum contains Acute Phase Proteins (APPs) capable of fighting bacterial infections. The presence of APPs, haptoglobin (Hp), C-reactive protein (CRP) and serum amyloid A (SAA) were identified in marsupial genomes, and expression of Hp and CRP were confirmed in phascogale tissues from birth to adulthood using PCR. Commercially available kits were used to determine the levels of Hp and SAA. Results indicate that female phascogales had higher Hp protein levels compared to males, and SAA protein levels in both males and females indicated that all phascogales sampled were clinically healthy at the time serum samples were collected.

Both complement proteins and APPs contribute to the antimicrobial properties observed in serum. The introduction of adult male and female phascogale serum to bacteria resulted in the growth inhibition of *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Staphylococcus aureus*. The effectiveness of antimicrobial properties in serum was dependent on the duration serum was introduced to bacteria at 37°C. Growth

inhibition was highest for *K. pneumoniae* and lowest for *P. aeruginosa*. All four bacteria exhibited growth inhibition, confirming the antimicrobial function of phascogale serum.

The results of this thesis have contributed to marsupial immunological research. Not many studies have focussed on the marsupial complement system; however this thesis has demonstrated the presence, function and effectiveness of the complement system in marsupials, and some initial research on complement in marsupial young. Marsupial young have no adaptive immune system, yet complement components were still expressed in 1-day old phascogales. In addition, the expression and levels of APPs in adult red-tailed phascogale serum have now been reported. This thesis shows that a greater emphasis should be placed on marsupial complement research, and other complement-associated components. Further research will enhance our understanding of the importance the marsupial complement system, and diseases associated with it.

CHAPTER ONE

INTRODUCTION

1.1 MARSUPIALS AS MAMMALS

All mammals have vertebrae, hair, mammary glands, and are endothermic (Vaughan, Ryan & Czaplewski 2013). Mammals are divided into three Orders; eutherians ("placentals"), metatherians (marsupials) and prototherians (monotremes) (Goodstadt et al. 2007), and they differ in their reproductive strategies. Eutherians and marsupials have a placenta, which has the ability to channel maternal blood flow to the foetus (Reilly & White 2003; Wildman 2011), and marsupials differ from eutherians because they give birth to comparatively underdeveloped young when compared to eutherians (Jackson 2003). Monotremes, unlike the other two mammalian classes, are egg-laying mammals (Graves 1996) and are limited to one extant platypus species (*Ornithorhynchus anatinus*) (Shaw 1799) and two extant echidna Genera; the short-beaked echidna (Genus *Tachyglossus*) (Griffiths 2012) and long-beaked echidnas (Genus *Zaglossus*) (Flannery & Groves 1998).

Extant marsupials inhabit the Americas and Australasia (Tyndale-Biscoe 2005). The two geographical groups of marsupials diverged more than 60 million years ago (Black et al. 2012). Since then, Australian marsupials have gradually developed to acclimatise to the Australian environment resulting in evolutionary differences between American and Australian marsupials (Tyndale-Biscoe 2005). Australian marsupials have a greater diversity compared to American marsupials (Ashwell 2010), and are classified into four subclasses, while American marsupials have three subclasses (Figure 1.1) (Hunsaker 2012).

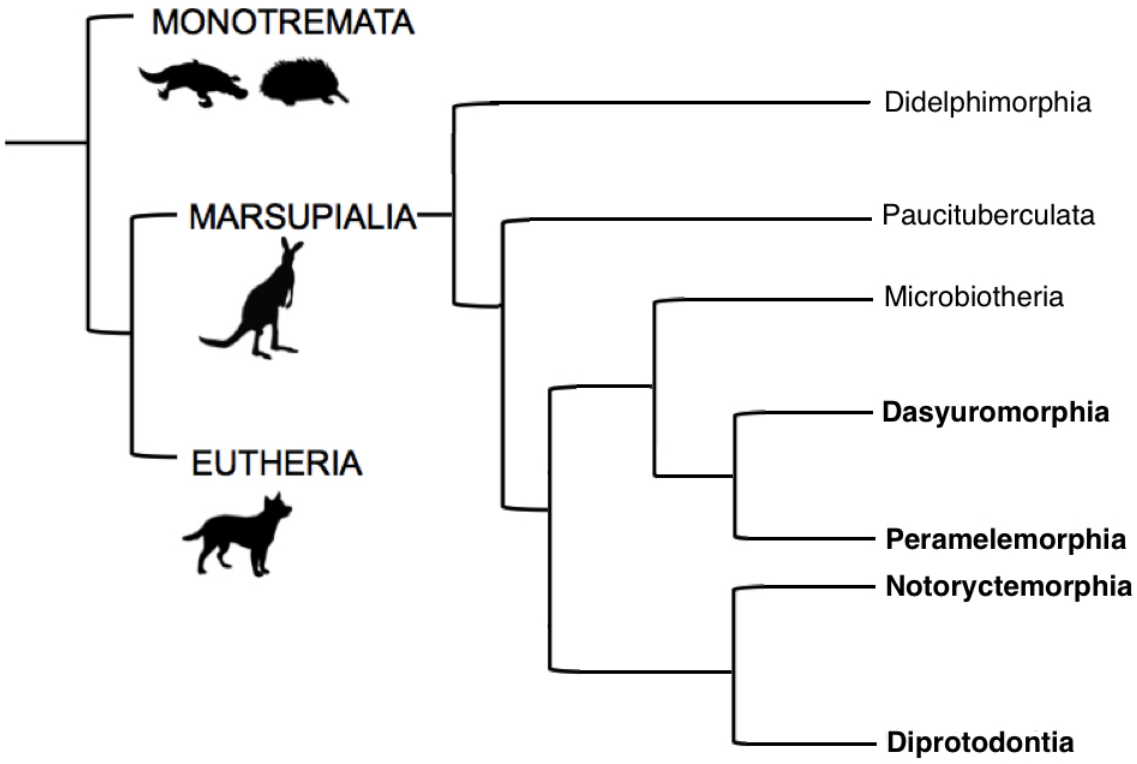

Figure 1.1 Cladogram representing Eutheria, Marsupialia and Monotremata, focussing on the Marsupialia group. The marsupials have two primary divisions: American and Australian (bold), which have diverged from each other due to a variety of environments and habitats.

One of the differences between marsupials and other mammals is their reproductive structures. Marsupials have two vaginae, two uteri, two oviducts, and between 10-23 chromosomes with one pair of sex chromosomes (Tyndale-Biscoe 2005). Marsupials also have a lower body temperature and metabolic rate compared to their eutherian counterparts (Tyndale-Biscoe 2005). Unlike eutherians, the development of marsupial pouch young *in utero* will only reach a certain state of development needed to pass through the urogenital opening, leaving the young exposed to a non-sterile external environment at the mother's teat after birth (Russell 1982). The survival of pouch young is heavily dependent on passive acquired immunity, where immunity of marsupial young is obtained from the mother (reviewed in Edwards et al. 2012). To receive maternal milk, marsupial young have to climb

to the mother's teat, and they do so by having well developed forefeet (Strahan 2004). Once attached, young then undergo a lengthy and complex lactation period during which physical and immunological development occurs (reviewed in Old & Deane 2000). The young are provided with early and late lactation proteins via their mother's milk, each of which has its own immunological functions (Joss et al. 2007; Lefèvre et al. 2007). Both early and late lactation proteins are unique to marsupial milk and have not been identified in eutherian milk, hence they are believed to be very important for the development of marsupial young (Lefèvre et al. 2007).

Suckling usually occurs on the maternal teat inside a 'marsupium' or 'pouch'. The 'pouch' is anatomically unique among the different marsupial species, for example, they can be deep or virtually non-existent, and can be forward or backward facing (Tyndale-Biscoe 2005). The presence of a deep pouch is beneficial because active secretions may be released to immunologically protect pouch young and control humidity and warmth (Edwards & Deakin 2013). "Pouchless" marsupials are reported to have tough skin (Saunders et al. 1989), providing extra protection in marsupial newborns. "Pouchless" marsupials, such as the gray short-tailed opossums (*Monodelphis domestica*), have pouch young that attach to the teats located on the underbelly. All marsupial young are exposed to the external environment throughout the prolonged lactation period, a factor beneficial to marsupial studies, as young can be observed and are easily accessible for experimental manipulation (Brunjes, Jazaeri & Sutherland 1992).

1.1.2 Red-Tailed Phascogale As A Study Species

Red-tailed phascogales are arboreal and insectivorous dasyurids (Bradley 1997) that are distinguished from other dasyurid species by their red-brown tail with brush-like hairs at the

tip (Figure 1.2) (Strahan 2004). Red-tailed phascogales are an ideal study species because they are small and captive populations are relatively easy to maintain (Foster et al. 2006). They also have an annual breeding season spread over three months, which can be used to introduce fertile males and females in one enclosure to ensure successful breeding (Foster et al. 2008).

Figure 1.2 A male (left) and female (right) red-tailed phascogale (Ong, 2015).

The red-tailed phascogale breeding season occurs around July each year (Bradley 1987). They become sexually mature at 11.5 months of age (Foster et al. 2008). After the breeding season, free living males exhibit semelparity (Bradley 1987). Before death, males suffer from an imbalance of glucocorticoid levels, and succumb to haemorrhagic ulceration due to immunosuppression (Bradley 1997; Bradley 1990). However, while free living males will experience synchronised mortality (Fisher et al. 2013), captive males will continue to survive but are reproductively senescent (Bradley 1997; Foster 2008; Stannard et al. 2013). This pattern has been observed in other captive semelparous males (Schmidt et al. 2006). Despite mortality, semelparity does provide an evolutionary advantage as the increase of glucocorticoid hormones before mating encourages the breakdown of proteins, allowing for

energy storage. The energy storage is then used for the annual breeding season which occurs during winter when food is scarce (Lee & Cockburn 2008).

Unlike males, female red-tailed phascogales can live for more than one breeding season and some have bred for at least four seasons in captivity (Stannard et al. 2013). Females give birth to supernumerary young, making it possible for a male-biased litter (Foster et al. 2008). Male-biased litters are advantageous as males are no longer fertile (captive) or die (free living) after their first breeding season, and a higher male population in the next generation ensures population increase (Foster & Taggart 2008).

Free living red-tailed phascogales have been internationally listed as endangered (Jackson 2003). Threats to red-tailed phascogale populations include destruction of habitat for agriculture, fire (Bradley 1997; Foster et al. 2006) and predation by invasive species, such as feral cats and foxes (Baillie et al. 1996). Their current distribution is limited to coastal woodland areas in south-western Western Australia (reviewed in Short & Hide 2012). In 1996, the red-tailed phascogale was presumed extinct in New South Wales (Dickman et al. 2001) and in 2008, the population was estimated at less than 10,000 mature animals, Australia-wide (Friend 2012). Despite phascogale habitats being well-distributed, they are fragmented due to intensive agriculture and habitat clearance (Short & Hide 2012). At present, the red-tailed phascogale population has remained steady, but undergoes fluctuations depending on rainfall (Friend 2012). There are conservation efforts in place to increase the number of phascogales in captivity so that they can be released back into the wild (Foster et al. 2006). A successful introduction occurred in 2009, when individuals sourced on farmland were introduced to the Wadderin sanctuary in Western Australia (Short & Hide 2015). After five years, phascogales are seen utilising hollows in trees and nest boxes in the sanctuary and population numbers have significantly increased (Short & Hide 2015).

The red-tailed phascogale is also an important study species because it is a dasyurid. Dasyurids are a family of marsupials that are distinguished through their four pairs of upper and three pairs of lower incisors, which separates the carnivorous from herbivorous marsupials (Morton, Dickman & Fletcher 1989). Although red-tailed phascogale express male semelparity, there are some that do not as their reproductive strategies vary depending on species (Lee, Bradley & Braithwaite 1977). Besides the red-tailed phascogales, other dasyurids, including the Tasmanian devil, Alexandria false antechinus (*Pseudantechinus mimulus*) and Northern quoll (*Dasyurus hallucatus*), are also endangered or vulnerable, and are experiencing population declines due to their sensitivity towards their environment (Finnie 1988). Even though not all population declines are attributed to the environment, some of it is still due to immunological incompetency (Morris et al. 2015).

Marsupial immunological research is fundamental for the investigation of immune mechanisms used in protection against diseases. Since immunology plays a huge role in the survival of a species, it is crucial that we enhance our knowledge on the marsupial immune system, and develop a profound level of understanding of these unique mammals.

1.2 MARSUPIAL IMMUNITY

The marsupial immune system is unique, with most immunological differences attributed to the different developmental stages of neonates in the different mammalian Orders. Like monotremes (reviewed in Kuruppath et al. 2012), marsupials are born with no adaptive immune system because of underdeveloped immune tissues, whereas eutherian neonates are born with a well-established adaptive immune system (Old & Deane 2000). Marsupial young likely rely heavily on passive acquired immunity, such as maternal milk and

pouch bacteria, as soon as they are born before developing their own adaptive immune system during pouch life (Adamski & Demmer 2000).

Marsupials have proven to be genetically and immunologically challenging to study due to a multiplicity of restrictions, including the lack of marsupial antibodies specific for tissue antigens (Hemsley, Canfield & Husband 1995) and the lack of publicly available marsupial genomes prior to 2007. Despite limited resources, marsupial immunologists have successfully made some comparisons between the marsupial immune system and other vertebrate immune systems, confirming its complexity and evolutionary position. For example, Cisternas and Armati (2000) found the presence of T- and B-cell markers and antigen presenting-like molecules in a marsupial, and Baker, Gemmell and Gemmell (1999) reported the distribution of T- and B-cells during marsupial development.

Fortunately, there are now four marsupial genome sequences available from marsupials in four different families. Marsupials with sequenced genomes include the gray short-tailed opossum (Mikkelsen et al. 2007), tammar wallaby (*Macropus eugenii*) (Renfree et al. 2011), Tasmanian devil (*Sarcophilus harrisii*) (Murchison et al. 2012) and koala (*Phascolarctos cinereus*) (Johnson et al. 2014). Annotated sequences of the gray short-tailed opossum, tammar wallaby, and Tasmanian devil (and a monotreme, platypus) can be acquired using GenBank (Benson et al. 2013) and Ensembl (Cunningham et al. 2015), making the comparisons between eutherian and non-eutherian gene sequences possible. Koala DNA and protein sequences are on the Koala Genome Consortium website (Johnson et al. 2014) (http://koalagenome.org).

Genome availability has enabled immunological sequences to be more readily identified in marsupials (Wong, Papenfuss & Belov 2011), and deepened the understanding of marsupial immunology. Collectively, all four marsupial genomes can be used to identify

differences between marsupial and eutherian genomes. However, despite advances in marsupial immunological research, the current understanding of the marsupial immune system remains largely rudimentary when compared to eutherians. Even after the release of the marsupial genomes, not all immune genes have been successfully identified and characterised in annotations, leaving gaps in our knowledge. Using eutherian genomes to identify certain genes, such as cytokines, have proven difficult because of the evolutionary divergence between eutherians and marsupials (Morris, Wong & Belov 2010).

1.2.1 MARSUPIAL YOUNG IMMUNITY

At birth, marsupial pouch young do not produce their own adaptive immune response, presumably due to a lack of mature lymphocytes (reviewed in Old & Deane 2000). When compared to eutherians, marsupials are born equivalent to an eight-week old human embryo (Block 1964), and rely on maternal milk for proteins and immunological components, including immunoglobulins (Joss et al. 2007), cathelicidins (Joss et al. 2009), cytokines (Young et al. 1997) and in some cases, the protection of the maternal pouch (Baudinette et al. 2005).

As marsupial young age, their immunological development begins to take shape. Marsupials are born with no mature lymphoid tissues. However, when young are two days old, they have lymphocytes in the thymus, which migrate around the body as they continue to develop (reviewed in Old & Deane 2000). Passive acquired immunity and the development of marsupial young's own immunity aid in the healthy growth and survival of young marsupials.

1.2.1.1 PASSIVE ACQUIRED IMMUNITY

Studies have found that prior to suckling; marsupial young lack the ability to induce specific immune responses. Mature lymphoid tissues are absent in the young of Virginia opossums (*Didelphis virginiana*; (Rowlands & Dudley 1968), tammar wallabies (Basden, Cooper & Deane 1996; Deane & Cooper 1984), quokkas (*Setonix brachyurus*) (Bell, Stephens & Turner 1974; Yadav, Stanley & Waring 1972) and common brushtail possums (*Trichosurus vulpecula*) (Ramadass & Moriarty 1992). Once born and attached to the teat, marsupials undergo a lengthy lactation period providing immune protection to their young, which is unnecessary for eutherian young as they develop some of their own innate and adaptive immunological strategies *in utero* (Sasaki et al. 2004; Shao et al. 2005).

There are three stages of marsupial lactation known as the early, mid and late lactation phase. Increased expression of immune components in the milk has been reported for early and late lactation phases in the tammar wallaby (Daly et al. 2007) and common brushtail possum (Adamski & Demmer 2000). Immune components secreted by the mammary glands include immunoglobulin heavy and light chains, polymeric Ig receptor, J chain and neonatal Fc receptor. These molecules all differ in expression levels during two of the lactation phases (early and late) to aid immunity at birth and also before leaving the pouch (Daly et al. 2007). Immunoglobulins IgA, IgG, IgM, IgE, Igκ and Igλ are also expressed throughout lactation (Daly et al. 2007) with IgA being the predominant immunoglobulin (Deane, Cooper & Renfree 1990). These results are comparable to a study by Adamski, King and Demmer (2000), that also found two of the three lactation phases had increased expression of secretory IgA and transferrin (early lactation phase), and IgG and transferrin (late lactation phase) in the common brushtail possum. These marsupial lactation studies indicate that milk proteins produced by marsupials contribute toward the survival of young marsupials.

The pouch also likely contributes to the immunological defence of marsupial pouch young. Marsupial pouches secrete maternally-derived antimicrobial compounds before birth, and presumably provides passively acquired immunity to pouch young (Ambatipudi et al. 2008; Bobek & Deane 2001). At the time of birth the pouch microflora changes, as evidenced in quokkas (Charlick et al. 1981), koalas (Bobek & Deane 2001), tammar wallabies (Old & Deane 1998) and common brushtail possums (Deakin & Cooper 2004). The antimicrobial substances found in pouch washes exhibit antimicrobial activity against *Escherichia coli* (Bobek & Deane 2001). Species such as tammar wallabies, with deep moist pouches, will also clean their pouch prior to the birth of pouch young through licking. Physically cleaning the pouch by licking likely decreases the number of harmful bacteria in the pouch via the action of salivary lysozymes and immunoglobulins (reviewed in Edwards & Deakin 2013; Old & Deane 1998).

Pouch young skin also likely contributes to protection from pathogens. Coutinho et al. (1995) found epidermal Langerhans cells (antigen-presenting cells) in the skin of Brazilian white-bellied opossum (*Didelphis albiventris*) pouch young, and Lyne (1970) found melanocytes protecting the epidermis of common brushtail possums at two to 100 days postpartum, suggesting that marsupial pouch young may have some of their own external immune protection right after birth. Other antimicrobial compounds including dermicidin and β-lactoglobulin, are released by epithelial cells and sweat glands lining the pouch, both of which are presumably involved in fighting Gram-negative bacteria (Ambatipudi et al. 2008).

Antimicrobial compounds have also been identified in marsupial milk. The expression of tammar wallaby cathelicidins, *MaeuCath1-7* present in tammar wallaby milk secretions (Wanyonyi et al. 2011), and *MaeuCath8* have been reported in developing tammar wallaby spleen, thymus, gastrointestinal tract, skin and liver tissues (Carman et al. 2009). The

expression of *MaeuCath1* has also been discovered in various mucosal organs in tammar wallaby pouch young, including the liver, spleen, bone marrow, skin, lungs and gut (Daly et al. 2008). Recently, four cathelicidins, labelled as *Cath 1, 2, 6* and *7*, have been reported as present in Tasmanian devil milk (Hewavisenti et al. 2016). The results of this study not only reaffirm the role of cathelicidins in the immune defence of pouch young, but also the effectiveness of immune transfer from mother to pouch young.

1.2.1.2 DEVELOPMENT OF MARSUPIAL IMMUNITY

During marsupial development, the thymus is the first specialised primary lymphoid organ to develop (Borthwick & Old 2016; Old & Deane 2000). The thymus has several important immunological roles, including T-cell maturation. The removal of the thymus gland from marsupial young results in reduced T- and B-lymphocyte responses (Miller et al. 1965), which have previously been linked to the complement system. The marsupial thymus is also unique in terms of anatomical location with most diprotodonts possessing two thymuses; a thoracic and cervical thymus, and polyprotodonts only possessing a thoracic thymus (Symington 1898) (reviewed in Borthwick, Young & Old 2014). The gene expression of the thoracic and cervical thymuses in tammar wallabies were reported to have similar gene expression to eutherian thymuses, which included genes regulating T-cell differentiation and immature T-cell proliferation (Wong et al. 2011). As marsupial young continue to develop, the thymus involutes and is replaced with fatty deposits (Cisternas & Armati 1999), similar to other mammals (Aspinall & Andrew 2000).

In comparison to eutherians, the timing of the appearance of some immune cells in marsupials differs, partly because marsupials are born at an earlier stage of development compared to eutherians. Haematopoiesis in marsupials continues in the liver after birth

(Borthwick & Old 2016; Old 2016), and the role of haematopoiesis is then taken over by the bone marrow, as occurs in eutherians (Basden, Cooper & Deane 1996; Block 1964). This pattern of development in marsupials is different when compared to eutherians, as eutherian mammals are born with functioning haematopoietic bone marrow, and the liver takes over gastrointestinal functions at birth (reviewed in Belov et al. 2013).

Haematopoiesis involves the production of T- and B-cells. In relation to lymphocyte development, the expression of cluster of differentiation molecules (CDs), CD8α and CD8β, are found in immune tissues of developing tammar wallabies from birth to 120 days (Duncan, Nair & Deane 2009). Some CDs are molecules that are important for antigen recognition; CD8 T-cells express CD8, which binds to antigen-associated Major Histocompatibility Complex (MHC) Class I molecules for the appropriate immune response (Janeway et al. 2008). CD8α and CD8β expression is aligned with the maturation of lymphoid tissues in young (Duncan, Nair & Deane 2009), indicating the development of their immunity as they mature into adults. CD8 is an essential molecule of cytotoxic T-cells and plays a pivotal role in the recognition and elimination of pathogens (reviewed in Chavez-Galan et al. 2009).

More recently in 2012, Wang, Sharp and Miller (2012) discovered B-cells in gray short-tailed opossums are initiated with CD79a and CD79b during the last 24 hours of gestation, suggesting the early development of immune protection in a marsupial pouch young prior to birth. This protection is further developed after birth as CD79a and CD79b transcripts have been found in gray short-tailed opossum and tammar wallaby in various organs after birth (Duncan et al. 2010; Old and Deane 2003).

By the time marsupial young are weaned, they have developed the ability to mount their own immunological response, including a functional adaptive immune system (reviewed in Old & Deane 2000). Lymphoid tissues in red-tailed phascogales, including thymus, bone

marrow, lymph node, spleen, liver and gut-associated lymphoid tissue (GALT), will also reach histological maturity before the young become independent (Borthwick & Old 2016).

1.2.2 Adult Marsupial Immunity

By the time marsupial young mature into adults, they are well equipped to mount immune responses, and other critical immune components, that are comparable to eutherians, have been identified. Immune tissues, such as the spleen and lymph nodes, have been fully developed and are directly involved in various immune responses (reviewed in Belov et al. 2013; Cisternas & Armati 1999).

Marsupial spleen and lymph nodes are major producers of cytokines during an infection (Harrison & Wedlock 2000). Cytokines are molecules involved in mediating immune responses, contributing to inflammatory, anti-inflammatory and cell-mediated immune responses (Ellis 2015). The identification of cytokines in marsupials has contributed to research on tumour suppression, thymus development and infectious diseases (Morris, Wong & Belov 2010). For example, one cytokine, interleukin-6 (IL-6), is mainly responsible for pro-inflammatory reactions and regulates the innate immune system in eutherians (reviewed in Kimura & Kishimoto 2010). IL-6 has been identified in several marsupials (Alsemgeest, Old & Young 2013; Borthwick et al. 2016; Morris & Belov 2013). It was expressed in lymph nodes and activated leukocytes of tammar wallabies, and is structurally similar to eutherian IL-6, indicating the possibility of similarities between the function of IL-6 in eutherians and marsupials (Alsemgeest, Old & Young 2013). However, further genetic analysis reported low amino acid sequence identity when compared to human IL-6, suggesting evolutionary divergence (Alsemgeest, Old & Young 2013).

The expression of IL-6 has also been reported in the red-tailed phascogale (*Phascogale calura*), kultarr (*Antechinomys laniger*) and stripe-faced dunnart (*Sminthopsis macroura*) (Borthwick et al. 2016). When analysed, IL-6 expression was significantly higher in lung and spleen tissue compared to heart, muscle, liver and kidney tissue of the adult red-tailed phascogale (Borthwick et al. 2016). Other interleukins have also been identified in the tammar wallaby (Young 2011; Young & Harrison 2010), and some other marsupial species, including the common brushtail possum (Wedlock, Aldwell & Buddle 1998; Wedlock et al. 1999) and koala (Mathew et al. 2013; Mathew et al. 2014).

The expression of IL-6 is particularly important, as it shows that IL-6 is being utilised in the marsupial immune system. In humans, it has been reported that IL-6 and IL-8 levels increased after complement activation (Rus, Vlaicu & Niculescu 1996), indicating that complement activation mediates the expression of certain interleukins. Later, it was reported that the incubation of Complement Complement 1, subcomponent q (C1q) with endothelial cells resulted in an upregulation of both IL-6 and IL-8 (van den Berg et al. 1998). C1q is a complement component involved in the activation of the Classical Complement pathway (reviewed in Sarma & Ward 2011); so it is likely that the Classical Complement pathway is involved in the upregulation of IL-6 and IL-8.

The complement system is involved in the innate immune system, but is also responsible for mediating adaptive immune responses. Previously, immune components have been divided into an immediate (innate) or acquired immune response (adaptive), however immune mechanisms such as the complement system broke down the barrier by having components involved in both immediate and acquired responses (Hoebe, Janssen & Beutler 2004).

B- and T-cells, responsible for antibody secretion, are both modulated by the complement system and are mainly involved in the adaptive immune system (Merle et al. 2015; Paul 2013). B-cells secrete cytokines and antibodies or immunoglobulins (Igs), which are an important part of the adaptive immune system. The Igs are antigen receptor antibodies that aid in the function of the innate immune system but also influence the adaptive immune system (Ochsenbein & Zinkernagel 2000). Immunoglobulins, IgA, IgE, IgG and IgM have been identified in marsupials (Miller 2010; Miller & Belov 2000). IgM and IgG activate the Classical Complement pathway in eutherians, and since both have been identified in various marsupial species (reviewed in Old & Deane 2000), could also be responsible for marsupial complement activation. Together with the complement system, Igs 'bridge' the gap between innate and adaptive immunity in all mammalian species (Macpherson, Geuking & McCoy 2011).

B-cells also express complement receptors that are able to interact with antigen surfaces and the opsonisation of a pathogen requires B- and T-cells to mount an adaptive immune response (Merle et al. 2015). There have already been studies in eutherians that show complement deficiencies leading to significantly decreased antibody responses generated by B-cells (Fischer et al. 1996) and inhibiting cytokine production by supressing the function of T-cells (Peng, McKenzie & Hwu 2016).

Upon complement activation, T-cells produce complement component 3, subcomponent a (C3a) and complement component 3, subcomponent b (C3b) after T-cell receptors are activated (Cardone et al. 2010). The production of C3a and C3b are important because it activates the production of interferon gamma (IFN-γ) (Le Friec et al. 2012). From this information, we discovered that complement component 3 (C3)-deficiency stops the

production of IFN-γ, a cytokine that activates macrophages, inhibits tumour growth and induce MHC Class II expression (Ting & Baldwin 1993).

The presence of mature B- and T-cells has been identified in various marsupials (Coutinho et al. 1995; Old & Deane 2003), and unlike eutherians, may have more diversity in terms of pathogen identification as they have a unique T-cell receptor (TCRμ) (Parra et al. 2007). T-cell receptors (α, β, γ and δ) that have been identified in both eutherians and marsupials are highly conserved, suggesting similar function and complexity between the two mammalian groups (Parra et al. 2008), and therefore is presumed to be related to the complement system similar to eutherians.

Despite having a well-equipped immune system, some marsupials still struggle with diseases to a point where their populations have significantly decreased. Although there could be many reasons why Tasmanian devils are susceptible to DFTD, researchers have found that it could be attributed to low genetic diversity within their immune genes. Morris et al. (2015) found low genetic diversity in immune genes including Toll-like receptors, however *MHC* has been the main focus (Cheng et al. 2012; Siddle et al. 2007), as the cancer cells causing DFTD are able to bypass the immune system by down-regulating cell surface *MHC* (Siddle et al. 2013).

MHC has been characterised in various marsupials, including the koala (Houlden, Greville & Sherwin 1996; Mayer et al. 1993), red-necked wallaby (*Macropus rufogriseus*) (Mayer et al. 1993) and the gray short-tailed opossum (Miska & Miller 1999; Stone et al. 1999). In these marsupial studies, we find that not all marsupials would suffer the same fate as the Tasmanian devils. The gray short-tailed opossum has been reported to have high *MHC* diversity, in contrast to the low *MHC* diversity in Tasmanian devils (Belov et al. 2006). The opossum has 13 MHC class I (Belov et al. 2006), four MHC class II (reviewed in Cheng,

Siddle & Belov 2010) and 48 MHC class III loci (Belov et al. 2006). When compared to *MHC* in eutherians, opossum *MHC* is similar in size and complexity. Despite the organisation of opossum MHC I, II and III genes being different from eutherian *MHC genes*, they are similar to non-mammalian *MHC genes*, suggesting that relocation of genes occurred after the divergence of marsupials and eutherians (reviewed in Cheng, Siddle & Belov 2010). It has also been reported that marsupial *MHC* is paralogous to eutherians (Belov, Lam & Colgan 2004), suggesting that the functionality of marsupial and eutherian *MHC* may be different.

The *MHC* is important for adaptive and innate immunity because of its ability to recognise foreign cells in the body (Ljunggren & Kärre 1990). The *MHC* does this by encoding various immune proteins, including complement proteins, allowing for a complete complement cascade for the elimination of pathogens (reviewed in Mayilyan 2012). Genetic diversity of *MHC* is essential because it allows for the detection of various pathogens (Janeway et al. 2001), and likewise the complement system is needed for cellular recognition of self and non-self in the body.

1.3 MARSUPIAL COMPLEMENT SYSTEM

The complement system is a group of proteins that work together to eliminate invading pathogens (reviewed in Sarma & Ward 2011). Complement was first known as a heat-sensitive immunological factor in serum (reviewed in Walport 2001), and was named because it complemented specific antibodies to lyse pathogens and foreign cells (Carroll 2004). Discovered by microbiologists in the late 19th century, complement proteins were functional between room temperature and 55°C. Four complement components were detected (Complement component 1-4) by 1920, and they were named according to the order that they were discovered, and not by their activation sequence (Ehrnthaller et al. 2011).

The complement system has been recognised as a major mediator of the innate immune system, and contributes toward modulation of the adaptive immune system (Cota & Midwinter 2009; Thiel et al. 2007). Once activated, the complement system causes an enzymatic reaction, or cascade of proteins, having been activated through various immunological processes (Zipfel & Skerka 2009). Currently, more than 30 complement proteins have been identified in eutherians, that all act together to support a functional complement system (Carroll 2004). The three main complement pathways are the Classical, Alternative and Lectin pathways – each with their own activation system (Figure 1.3) (Sarma & Ward 2011).

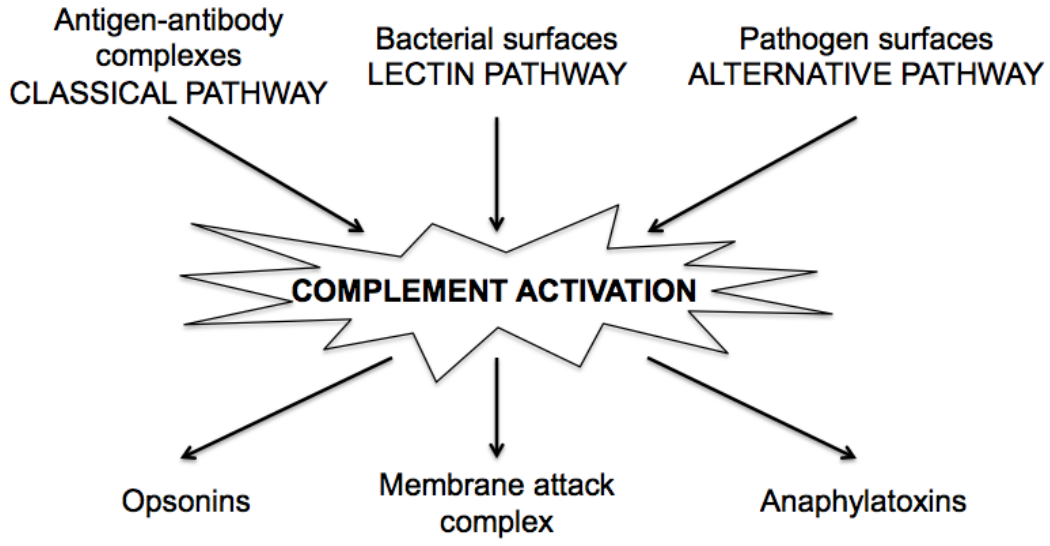

Figure 1.3 A simplified version showing the activation methods of classical, lectin and alternative pathways, leading to three different outcomes; the production of opsonins, anaphylatoxins and/or Membrane Attack Complex (MAC) (adapted from (Sarma & Ward 2011).

The complement system is well documented in eutherians, however the presence of a functional complement system has only been identified in two marsupial species

(Koppenheffer *et al.* 1998; Wirtz and Westfall 1967). Initially thought to only be involved in the innate immune system, the question of whether the complement system is also contributing to the adaptive immune system arose when researchers found B-cells attached to C3 (Nussenzweig et al. 1971). Later, it was found that complement receptors enhance B-cell immunity, and bind to opsonins (an effect of the complement system) to aid in phagocytosis (Kinoshita et al. 1990; Van Lookeren Campagne, Wiesmann & Brown 2007).

Wirtz and Westfall (1967) first described the presence of a functional complement system in a marsupial, the Virginia opossum, by successfully lysing sensitised sheep erythrocytes using adult opossum serum with the standard haemolytic assay. Using similar methods, Koppenheffer, Spong and Falvo (1998) described the presence of a functional Classical and Alternative Complement system in the gray short-tailed opossum. The detection of the Alternative Complement pathway is possible by replacing sheep erythrocytes with rabbit erythrocytes in a standard haemolytic assay. Both studies found that complement functioned well in opossums when the ionic strength was between 0.7 and 0.11 and at the optimal temperature for sheep erythrocyte lysis at ~30°C (Koppenheffer, Spong & Falvo 1998; Wirtz & Westfall 1967).

Other studies have found the presence of proteins involved in complement. Lynch et al. (1992) isolated a C3-like protein in the quokka, which had a similar structure to eutherian C3, suggesting a similar function. Baker et al. (2007) isolated the *C3 gene* from the bandicoot (*Isoodon macrourus*) using ESTs. The identification of C3 suggests a functional complement system because it is the central complement component in the three main pathways (Classical, Alternative and Lectin). Complement-like proteins have also been found in marsupial milk (Joss et al. 2009). Joss et al. (2009) identified 'Very Early Lactational Protein' (VELP) and a complement B protein in tammar wallaby milk, both with a similar

structure to mannose-binding lectin (MBL) and complement factor properdin (CFP), respectively.

So far, these are the only studies that have investigated the complement system in a marsupial. While there are other studies that have briefly indicated the presence of the complement system in a marsupial, complement has rarely been investigated in detail in marsupials. The function of the non-eutherian mammal complement system has been limited to opossums (Koppenheffer, Spong & Falvo 1998; Wirtz & Westfall 1967), and even though complement and complement-like proteins have been identified in other marsupials (Baker et al. 2007; Lynch et al. 1992), our knowledge of the marsupial complement system remains very limited. In addition, the expression of complement proteins has been largely unexplored. The complement system is more widely discussed in Chapter 2.

With four marsupial studies suggesting that the marsupial complement system is comparable to eutherians, there is now a possibility of obtaining complement sequences using marsupial genomes to aid further studies in this area. There is the possibility that marsupials rely on innate immunity during pouch life (Belov et al. 2006), and studying the expression of these complement genes in pouch young has now become a possibility.

1.4 AIMS

The complement system is a primitive immune function that has been found in invertebrates and vertebrates, including mammals (reviewed in Fujita, Endo & Nonaka 2004). Since the marsupial immune system has been proven to be complex but comparable to the eutherians (Belov et al. 2006; Belov et al. 2007), we expect to find the presence of a complement system in adult marsupials, as well as complement expression in immune tissues

and serum for immune protection. Comparative immunological studies have found that marsupial young are born with no adaptive immune system, unlike eutherians that have developed adaptive immunity due to longer gestation periods (Old & Deane 2000). However, the complement system bridges the innate and adaptive immunity, therefore it is predicted that the expression of complement proteins is present in marsupial young and is likely to play a role in immune defence prior to the development of a fully functional adaptive immune system. The complement system would then be fully functional once marsupial young are weaned and become adults.

From previous research, we know that the complement system is functional in two marsupial species, the gray short-tailed opossum and Virginia opossum. However, we do not know, in depth, if there are functional similarities between the different types of mammals. The aim of this thesis was to focus on non-eutherian mammal genomes and the complement system of a marsupial. By conducting the genomic survey, and investigating complement gene expression and performing complement assays, a comparison can be made between presence, expression and function of various complement components or pathways, and whether they are comparable to eutherians.

The comparative studies between non-eutherian and eutherian mammal immunity will allow us to make comparisons to other vertebrate immune systems, leading to a greater understanding of the phylogeny of the immune system. The work described in this thesis examines the non-eutherian mammal complement system, and its specific role in red-tailed phascogales. It improves our understanding of the developing immune system of marsupials, particularly Dasyurids, which may aid future studies and declining populations.

1.5 THESIS OUTLINE

The thesis is separated into six chapters (including this introduction and a discussion), focussing on the non-eutherian complement system. Five of the following six chapters are written as publications, and differ in format according to the journal they were submitted to. The following five chapters aimed to:

Chapter 2: Identify the key complement components that have been identified in humans, in non-eutherian mammals using available genomes.

Chapter 3: Determine stable reference genes suitable for use in expression studies in red-tailed phascogale immune tissues.

Chapter 4: Determine the expression of complement components specific to the Classical, Alternative and Lectin Complement pathways in adult and pouch young red-tailed phascogales.

Chapter 5: Determine the functionality of Classical and Alternative Complement pathways in red-tailed phascogales.

Chapter 6: Determine the antimicrobial activity of red-tailed phascogale serum.

1.6 CHAPTER 1 REFERENCES

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CHAPTER TWO

PRELIMINARY GENOMIC SURVEY AND SEQUENCE ANALYSIS OF THE COMPLEMENT SYSTEM IN NON-EUTHERIAN MAMMALS

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2.1. Chapter Outline And Authorship

Chapter 2 is a preliminary genomic survey and sequence analysis of the complement system in five different non-eutherian mammals. The species included in this study were the gray short-tailed opossum (*Monodelphis domestica*), tammar wallaby (*Macropus eugenii*), Tasmanian devil (*Sarcophilus harrisii*), koala (*Phascolarctos cinerius*) and platypus (*Ornithorhynchus anatinus*) as their genomes are publicly available. The releases of non-eutherian genomes have made it possible to identify complement sequences that have already been identified in eutherians. Confirming the presence of complement components will reinforce our knowledge that marsupials have the potential to use the complement system as a defence mechanism against pathogens.

The manuscript is jointly authored, where I am the primary author, conducted the bioinformatic analysis using the four genomes, and drafted the manuscript. Dr. Lauren Young contributed ideas and development of the manuscript and provided feedback on manuscript drafts. Associate Professor Julie Old conceived the study, supervised the development of the study, provided editorial feedback on manuscript drafts and is the corresponding author of the manuscript.

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2.2. INTRODUCTION

Mammals arose approximately 200 million years ago from "mammal-like" reptiles (Flight 2011) and today there are three extant classes of mammals (eutherians, marsupials and monotremes). Eutherians are born well-developed, while marsupials are born at an earlier stage of development and only have the necessary features to crawl from the vulva to the teat, where they attach and undergo continued maturation (Tyndale-Biscoe 2005). The third group, monotremes, are unique among mammals as they lay eggs (Warren *et al.* 2008; Wrigley and Graves 1988). The study of immune systems of different mammal classes is important from an evolutionary viewpoint, because of their different developmental stages at the time of birth or hatching.

The timing of divergence for these different mammalian groups is important as it resulted in different developmental stages. Monotremes diverged from therian mammals (eutherian and marsupials) approximately 166 mya (Renfree *et al.* 2009). Eutherians and marsupials then diverged approximately 160 mya (Luo *et al.* 2011), while the South American marsupial, the gray short-tailed opossum (*Monodelphis domestica*) and three Australian marsupials, the fat-tailed dunnart (*Sminthopsis crassicaudata*), tammar wallaby (*Macropus eugenii*) and common brushtail possum (*Trichosurus vulpecula*) diverged approximately 70 mya (Rens *et al.* 2001).

Until the last decade, all available mammalian genome sequences have been eutherian (Mikkelsen *et al.* 2007). The first high quality draft sequence from any marsupial was that of a female gray short-tailed opossum (Mikkelsen *et al.* 2007). The release of the opossum genome was followed by the release of the platypus (*Ornithorhynchus anatinus*; Warren *et al.*, 2008), and Australian marsupials, the tammar wallaby (Renfree *et al.* 2011), Tasmanian

devil (*Sarcophilus harrisii*; Murchison *et al.*, 2012) and koala (*Phascolarctos cinereus*; Hobbs *et al.* 2014). The availability of this sequence data has provided evolutionary insights into the genes related to the immune system of these non-eutherian mammals (Wong *et al.* 2006; Wong *et al.* 2011).

Before the release of these genomes, immune gene comparisons between eutherian and non-eutherian mammals were not possible unless laboratory-intensive homologous cloning techniques were utilised. Harrison and Wedlock (2000) isolated and cloned orthologues of tumour necrosis factor (TNF), lymphotoxins α and β, interleukin-1 β and interleukin-10 by designing consensus primers using the gene sequences of eutherian counterparts that had similar structure and functionality (Harrison and Wedlock 2000). However this was only possible for genes that were highly conserved across phylogenetic distances (Wakefield and Graves 2005) and was limited in scope, particularly with respect to the analysis of more complex pathways. Nonetheless many studies were undertaken on the evolution of marsupials over the last few decades providing a framework for more extensive analysis, once genomic data became available (Deakin 2012).

Genomes of gray short-tailed opossum and tammar wallaby have been used as model species to study the evolution of the mammalian immune system, particularly from a developmental perspective. Marsupials are born with no histologically mature immune tissues, and are therefore unable to mount specific immune defences against pathogens at birth (reviewed in Old & Deane, 2000 and Borthwick *et al.* 2014). Consequently, they are dependent on maternally derived strategies for immune defence, such as maternal milk and pouch antimicrobial secretions during pouch life, and it is likely that complement proteins play a significant role in pouch young immunity (Ambatipudi *et al.* 2008; Joss *et al.* 2009; Edwards and Deakin 2012). Previous protein studies have found that tammar wallaby milk

secretions from the mother include complement B protein (Joss *et al.* 2009) and very early lactation proteins (VELP), a protein similar to mannose-binding lectin (MBL) that aids in the activation of the lectin complement pathway in eutherians (Joss *et al.* 2007; Kuy *et al.* 2007).

The development of the immune system of monotremes is comparable to marsupial pouch young as monotremes are also born without histologically mature immunological tissues (Holland and Jackson 2002). Monotreme hatchlings go through a six to ten day incubation period (Grant 1995) and remain in the burrow for approximately 130 days (Holland and Jackson 2002), suckling on maternal "milk patches" (Whittington *et al.* 2008; Whittington *et al.* 2009), which provide innate immunity for the hatchlings in the form of bactericidal proteins, such as lysozyme (Hopper and McKenzie 1974) and transferrin (Teahan *et al.* 1991).

Overall, the complement system in eutherian mammals is well understood but very little is known about this system in non-eutherian mammals, despite the complement system being present in all vertebrates from bony fishes to mammals, and having a comparable complement system with respect to complement genes (reviewed in Nonaka and Kimura 2006). Complement consists of a group of serum proteins (both activators and inhibitors) that are part of the humoral immune system, but are also linked to adaptive immune responses. Mainly synthesised by the liver, complement proteins undergo proteolytic cleavage after activation, and remove foreign and apoptotic cells (Sarma and Ward 2011). In the past decade, there has been increased acknowledgement of the complement system and its role in both innate and adaptive immune strategies (Mollnes *et al.* 2007).

The eutherian complement system has several activation pathways — the most widely studied are the classical (antibody-activated), alternative (activated by microbial cell surface molecules) and lectin (microbial sugar)-binding pathways (Fig. 1). Although each pathway is

activated by a set of different molecules (outlined later in the text), they all share a central event, which is the downstream formation of an important central complement component, complement component 3 (C3). C3 has specific binding sites that enable it to bind to other molecules, leading to its activation. C3 activation results in the exposure of intramolecular thioester bonds to its molecular surface. The molecular surface then forms a covalent bond with invading microorganisms, resulting in the activation of other complement components, ranging from C5 to C9, inducing cytolytic complex formations, leading to the formation of a membrane attack complex (MAC). A pore in the membrane of an invading pathogen causes it to lyse, effectively puncturing the cell (Janeway *et al.* 2001).

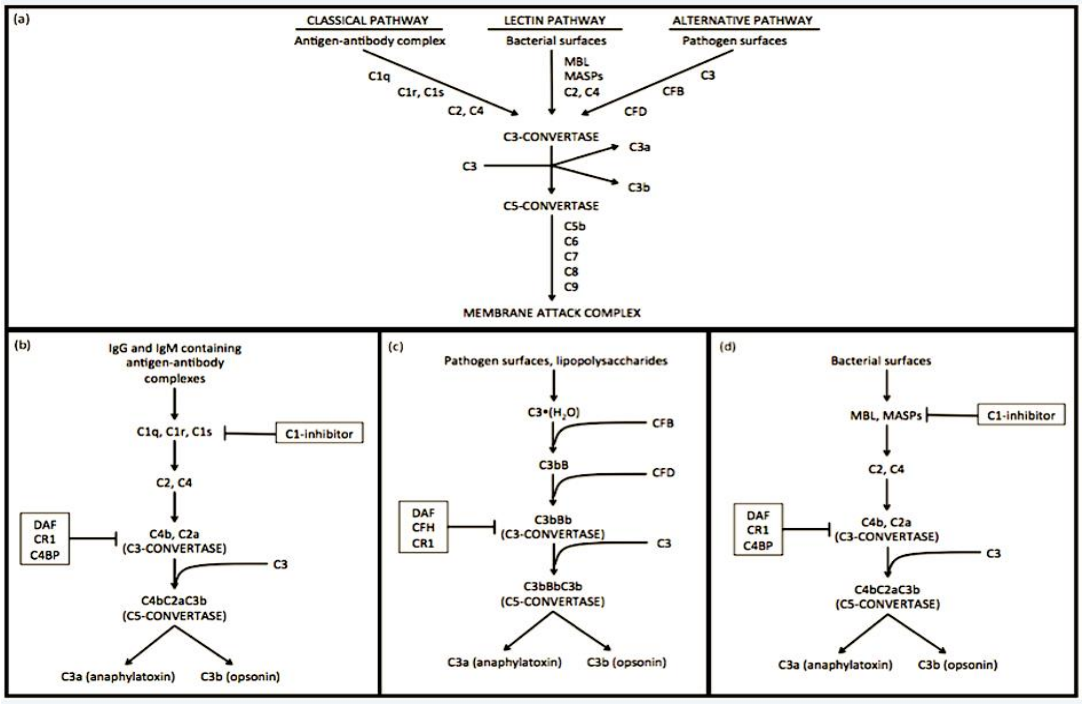

Figure 2.1 The main complement pathways of the complement activation system in humans. (A) A summary of the main complement pathways emphasising the common pathway and complement proteins in all three complement pathways: classical, lectin and alternative (adapted from Taylor *et al.* 1998). Detailed information on the three complement pathways are shown in: (B) the classical complement pathway (adapted from Sarma and Ward 2011), (C) the alternative complement pathway (adapted from Sarma and Ward 2011), and (D) the lectin complement pathway (adapted from Sarma and Ward 2011). Figures also show inhibitory molecules (boxed).

Aside from members of the direct complement pathway, there are complement inhibitors that mediate the complement response. These inhibitors modulate complement activation, which helps to limit damage to host tissues and prevent complement-related immune disorders. Both activation and inhibitory molecules associated with eutherian complement pathways are also important to study in the context of a genome-based complement survey (Janeway *et al.* 2001).

The presence of the classical complement pathway was inferred some time ago in the gray short-tailed opossum (Wirtz and Westfall 1967) and Virginian opossum (*Didelphis virginiana*; Koppenheffer *et al.* 1998) using well-recognised haemolytic functional assays that are commonly applied to eutherian systems. During this time period, a C3-like protein was also reported in a macropod species, the Australian quokka (*Setonix brachyurus*). This C3-like protein had similar structure and function to C3 in eutherian mammals (Lynch *et al.* 1993). More recently both the classical and alternate complement pathways have been reported for the red-tailed phascogale (*Phascogale calura*) (Ong *et al*. 2015).

In addition, with the application of molecular gene expression methods such as Expressed Sequence Tag (EST) investigations, Baker *et al.* (2007) confirmed the nucleotide expression of the C3 gene in thymus tissue in the gray short-tailed opossum. Since C3 is a central complement component for the complement system in eutherians, the study further supported the notion of complement being a part of the immune repertoire of marsupials, although there was clearly still much more to learn about the nature and complexities of the individual pathways in marsupials. We identify and compare the gene sequences for complement and complement-related components of four marsupials and a monotreme species using the complement system of humans (available from public databases) as a guide.

A comparison of C3 in all mammalian groups was also conducted using sequence alignments and motif comparisons.

2.3. METHODS

2.3.1. SEQUENCE IDENTITY

To identify the presence of complement genes common to both human and non-eutherian mammals the human genome database in GenBank (Benson *et al.* 2010; http://www.ncbi.nlm.nih.gov/genbank/) was investigated for key components of the complement family and related proteins, while non-eutherian sequences (except koala) were obtained from GenBank (Benson *et al.* 2010; http://www.ncbi.nlm.nih.gov/genbank/) and Ensembl release v78 databases (Flicek *et al.* 2013; http://www.ensembl.org). Specifically, we searched the genome data of human (*Homo sapien*, http://www.ncbi.nlm.nih.gov/genome/51), gray short-tailed opossum (GENBANK: AAFR00000000.3; ENSEMBL: GCF_000002295.2), tammar wallaby (GENBANK: ABQO000000000.2; ENSEMBL: GCA_000004035.1), Tasmanian devil (GENBANK: AEFK00000000.1; ENSEMBL: GCA_000189315.1) and the platypus (GENBANK: AAPN00000000.1); ENSEMBL: GCF_000002275.2). Koala complement sequences were obtained from the Koala Genome Consortium (KGC; Johnson *et al.* 2014; http://www.koalagenome.org/). Not all genes were investigated, as our study was limited to important members of the complement cascade, which are listed in Table 2.1. Non-eutherian complement components were aligned with their human homologues and the highest e-value, along with percent identity, are listed in Supplementary Material 2.1.

In an effort to identify non-eutherian gene homologues, complement gene sequences were extracted from the human genome, and similarity searches were performed using Basic

Local Alignment Search Tools (BLAST; Altschul *et al.* 1990); nucleotide BLAST (BLASTN) and protein BLAST (BLASTP), on the non-eutherian genomes (gray short-tailed opossum, tammar wallaby, Tasmanian devil, koala and platypus) available in GenBank, Ensembl and KGC databases. Where nucleotide or protein sequences have been identified for a particular gene and its corresponding nucleotide/protein sequences were not found, nucleotide sequences were derived using BLAT (Kent *et al.* 2002). The corresponding protein sequence was used as a query, and protein sequences derived using the translation tool, ExPASy (http://www.expasy.org/) using the corresponsing nucleotide sequence as the query. The translation of nucleotide to protein sequences, and vice versa, did not apply to koala sequences as access to full koala sequences is currently limited on the KGC. Gray short-tailed opossum sequences were also used to search the other non-eutherian genomes. The database origins of these genes are listed in Supplementary Material 2.2.

Table 2.1 The nucleotide/amino acid identity when compared to their respective human sequence. Nucleotide identities and amino acid identities are represented by normal and italic fonts, respectively.

Systemic	**Nucleotide/amino acid identity (%)**				
name	**Opossum**	**Tammar**	**Devil**	**Koala**	**Platypus**
C1QA	69, *61*	–	69, *55*	*75*	69, *56*
C1QB	71, *67*	–	72, *63*	*68*	–
C1QBP	76, *75*	80, *85*	78, *83*	*75*	–
C1QC	74, *71%*	74, *70*	74, *70*	*74*	73, *67*
C1QL2	77	79	76	86	–
C1QL4	85, *96*	86, *96*	83, *95*	85, *87*	90, *92*
C1QTNF1	81, *76*	83, *86*	79, *78*	*69*	84, *81*
C1QTNF2	78, *82*	78, *78*	80, *84*	*82*	80, *87*
C1QTNF3	85, *86*	–	86, *88*	*89*	81, *91*
C1QTNF4	–	–	84, *78*	*79*	79, *74*
C1QTNF5	81	85, *56*	82, *83*	81, *63*	86, *93*
C1QTNF6	76, *65*	72, *65*	72, *70*	76, *65*	76, *67*
C1QTNF7	80, *91*	85, *93*	84, *90*	82, *92*	76, *88*
C1QTNF8	69, *57*	72, *64*	68, *56*	*49*	–
C1QTNF9	77, *75*	–	76, *76*	77, *75*	74, *71*
C1RL	73, *58*	–	72, *56*	*58*	70, *59*

C1S	75, *70*	75, *71*	75, *71*	76, *71*	69, *67*
C2	–	–	–	*71*	71, *53*
C3	70, *66*	69, *66*	–	*79*	69, *63*
C3AR1	77, *55*	–	77, *52*	*52*	74, *48*
C4A	71, *67*	–	70, *66*	72, *67*	–
C4B	–	–	–	72, *71*	–
C4BPA	70, *49*	–	66, *50*	*49*	65, *46*
C4BPB	69, *49*	–	*42*	*49*	–
C5	74, *67*	76, *56*	74, *67*	*68*	71, *65*
C5AR1	66, *51*	–	70, *48*	*54*	67, *55*
C6	74, *67*	75, *62*	73, *67*	*69*	73, *59*
C7	72, *64*	71, *63*	73, *65*	*66*	65, *62*
C8A	65, *55*	68, *43*	67, *57*	*56*	76, *52*
C8B	68, *40*	–	–	–	67, *58*
C8G	66, *55*	–	65, *58*	*54*	69, *44*
C9	72	71	70	*85*	73
CD46	–	–	–	–	–
CD55	70, *55*	69, *59*	68, *51*	*50*	69, *51*
CD59	–	–	–	–	–
CFB	74, *68*	–	72, *68*	*69*	–
CFD	–	68, *68*	68, *68*	*70*	70, *66*
CFH	71, *48*	–	71, *36*	*47*	70, *29*
CFHR1	–	–	–	–	–
CFHR2	–	–	–	–	69, *59*
CFHR3	–	–	–	–	–
CFHR4	–	–	–	–	–
CFHR5	66, *37*	72, *56*	–	–	67, *42*
CFI	69, *53*	–	71, *57*	*58*	64, *52*
CFP	67, *61*	–	70, *64*	*61*	66, *58*
CPAMD8	74, *74*	73, *73*	74, *75*	74, *75*	–
C1R	74, *71*	77, *73*	72, *70*	*70*	71, *68*
CR1	–	–	72, *51*	–	67, *41*
CR2	68, *42*	66, *52*	68, *40*	*43*	67, *36*
CRP	69, *68*	–	69, *54*	*60*	72, *43*
FCN3	70, *51*	74, *66*	–	–	–
ITGAM	68, *60*	67, *61*	68, *61*	*62*	70, *56*
ITGAX	68, *61*	–	*60*	*61*	–
MASP1	82, *81*	81, *70*	81, *81*	81, *83*	80, *81*
MASP2	77, *72*	72, *61*	74, *94*	*67*	73, *65*
MBL2	66, *57*	–	–	*53*	–
SERPING1	71, *62*	–	72, *56*	*55*	70, *61*

2.3.2. Sequence Alignments and Motif Analysis

Nine complement components were chosen for sequence alignments using ClustalW (Thompson *et al.* 1994) because of their importance and specificity to the three main complement pathways (Table 2.1 and Table 2.2). Eutherian sequences used in the alignments for comparison with our non-eutherian sequences include pig (*Sus scrofa*), mouse (*Mus musculus*), dog (*Canis lupus familiaris*), cattle (*Bos taurus*) and sheep (*Ovis aries*). The chicken (*Gallus gallus*) sequence was included where possible to identify conserved sequence data across the vertebrate groups.

Table 2.2 Complement components that are specific to the classical, alternative and lectin complement pathway.

Complement pathway	Complement components
Classical complement pathway	C1q C1r C1s
Alternative complement pathway	CFB CFD CFP
Lectin complement pathway	MBL2 MASP1 MASP2

In many gene families, conservation of a pattern of amino acids is observed across phylogenetic boundaries, which can help to confirm the identity of putative genes and their translated proteins. Sequence motif analyses were conducted for the selected complement components (listed on Table 2.3) by inserting human and non-eutherian complement protein sequences into MOTIF (GenomeNet; http://www.genome.ad.jp/) using a PROSITE pattern notation. If protein sequences were not available for the gene in question, complement nucleotide sequences from GenBank or Ensembl were translated into predicted protein

sequences using ExPASy Translate Tool (http://www.expasy.ch/tools/dna.html) and inserted into MOTIF.

Table 2.3 Complement components chosen for sequence alignments and motif comparisons.

Complement pathway	Complement components
Classical complement pathway	Complement component 1, q subcomponent, A chain (C1qA) Complement component 1, q subcomponent, B chain (C1qB) Complement component 1, q subcomponent, C chain (C1qC)
Alternative complement pathway	Complement factor B (CFB) Complement factor D (CFD) Complement factor properdin (CFP)
Lectin complement pathway	Mannan-binding lectin serine protease 1 (MASP1) Mannan-binding lectin serine protease 2 (MASP2)
All three pathways	Complement component 3 (C3)

We focussed on five main motifs; epidermal growth factor (EGF) (Reid and Day 1989), integrin β (Holmes and Rout 2011), anaphylatoxin (Klos *et al.* 2009; Sontheimer *et al.* 2005), von Willebrand factor (vWF) (Hunt and Barker 1987) and defensin (Lynn and Bradley 2007) due to their well-recognised presence in known complement family molecules. The sequences in alignments had various lengths, depending on the sequence provided by GenBank and Ensembl, but had similar functionality according to motif comparisons. Functionally important residues were labelled and residues with >70% nucleotide identity were highlighted. The length of each complement component from human, gray short-tailed opossum, tammar wallaby, Tasmanian devil and platypus were documented along with the percent identity and e-value of sequences from non-eutherian mammals in comparison to human using whole sequences from respective databases.

2.3.3. The Central Complement Component, C3

Further sequence alignments of C3 were conducted to analyse the quality of the predicted genomic sequences, by comparing them to their corresponding expressed C3 genomic sequences in the gray short-tailed opossum and tammar wallaby that were found in GenBank. The predicted and expressed C3 genomic sequences for both marsupials were obtained from GenBank and Ensembl.

A phylogenetic tree was also constructed using expressed C3 protein sequences from non-eutherians; gray short-tailed opossum (GENBANK: XP_003340773.1), tammar wallaby (GENBANK: AAW69835.1) and northern brown bandicoot (*Isoodon macrourus*)(GENBANK: ABC84125.1; partial), and eutherians; human (GENBANK: AAA85332), cattle (GENBANK: AAI12453), pig (GENBANK: AAG40565) and mouse (GENBANK: AAH43338). Predicted C3 protein sequences were included as they are non-eutherian mammals and were found to be "predicted" in GENBANK (Platypus; GENBANK: XP_007670891.1). The koala C3 protein sequence was not included in the phylogenetic tree as access to sequence was not available through the KGC database. The phylogenetic tree was constructed using Geneious, version R6.1 by Biomatters (http://www.geneious.com). The sequence alignment for construction of the C3 phylogenetic tree was conducted using Geneious with the Blosum62, alignment score matrix, with a gap open penalty score of 12, and gap extension penalty score of 3. The unrooted morphological phylogenetic tree was constructed using the neighbor-joining tree build method, and the sequences were evolved according to the Jukes-Cantor model.

2.4. RESULTS

2.4.1. SEQUENCE IDENTITY, ALIGNMENTS AND MOTIF IDENTIFICATION

Of the 57 complement and complement-related genes chosen from the human genome and compared to sequences in the gray short-tailed opossum, tammar wallaby, Tasmanian devil and platypus genomes, 46 homologues were identified in the genome of the gray short-tailed opossum, 27 in the tammar wallaby, 44 in the Tasmanian devil, 47 in the koala and 40 in the platypus (Table 2.1). No additional genes were identified using pairwise alignments in Ensembl. Fig. 2.2 gives a visual representation of the proportion of human complement proteins found in comparison to non-eutherian complement proteins. In addition, complement components were also divided into different classes of the complement system.

We found that complement components such as C3, C1q, CFP, MASP1 and MASP2, that were particularly specific to either classical, alternative or lectin complement pathway, were identified in all non-eutherian mammals investigated (Table 2.2). Sequence motif analyses on complement components C3, C1qA-C, CFD, CFB, CFP and MASP1-2 showed that the common motif identified was epidermal growth factor (EGF). Other common motifs identified in relevant genes in this study include the von Willebrand factor, type C (vWFC) and anaphylatoxin (Supplementary Material 2.3).

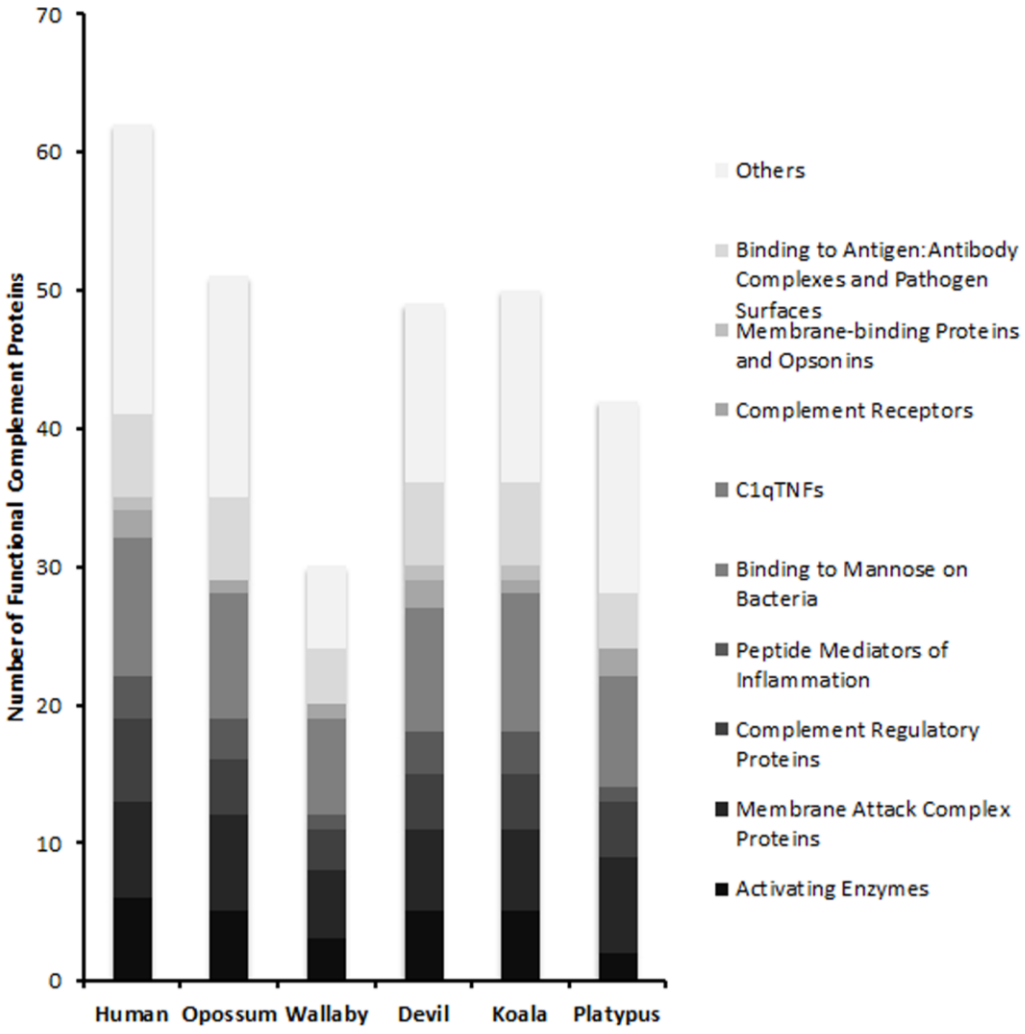

Figure 2.2 The number of complement components in different classes of the complement system. Complement proteins that did not fall into any of the functional protein classes are listed as 'other'.

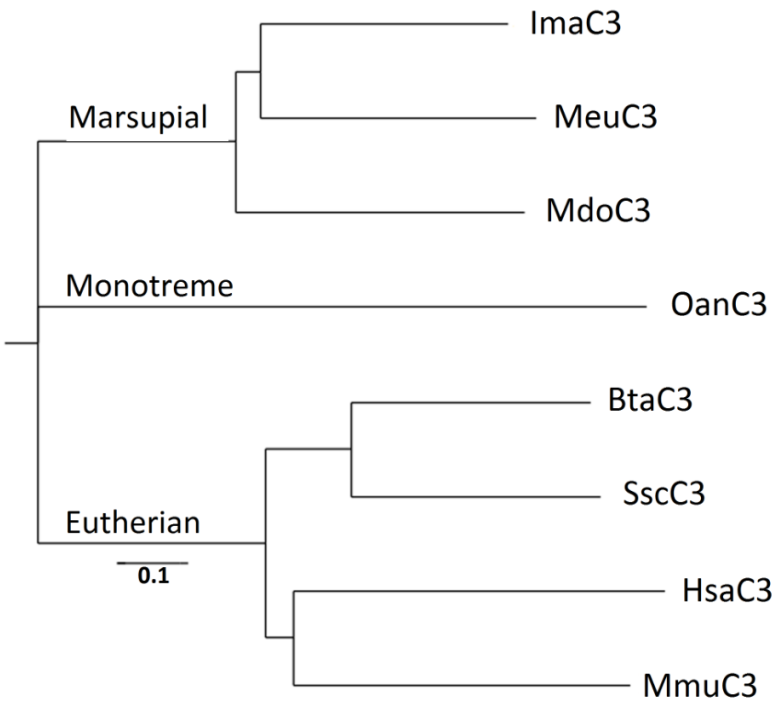

Figure 2.3 Morphological phylogenetic tree developed using C3 protein sequences from GenBank protein database except for gray short-tailed opossum and echidna. All the C3 sequences are expressed sequences, except for the Tasmanian devil and platypus. The units of branch length represent the number of substitutions per site. The sequences are abbreviated using the first letter from the genus and the first two letters of the species name followed by the gene name. Protein sequences used in this tree were from the GenBank protein database and include: Human (Hsa; *Homo sapiens;* GENBANK: AAA85332), gray short-tailed opossum (Mdo; *Monodelphis domestica*; GENBANK: XP_003340773.1), tammar wallaby (Meu; *Macropus eugenii;* GENBANK: AAW69835.1), northern brown bandicoot (Isa; *Isoodon macrourus*; GENBANK: ABC84125), platypus (Oan; *Ornithorhynchus anatinus*; GENBANK: XP_007670891.1), cattle (Bta; *Bos Taurus;* GENBANK: AAI12453), pig (Ssc; *Sus scrofa;* GENBANK: AAG40565) and mouse (Mmu; *Mus musculus*; GENBANK: AAH43338).

Functional conserved sequences have been detected in the multiple sequence alignment of C3 from a range of species through the sequence alignments (Supplementary Material 2.4). The phylogenetic tree using predicted and expressed protein C3 sequences from eutherian and non-eutherian mammals indicates clearly that the C3 gene is an orthologue that has diverged in the three different mammal groups; eutherians, marsupials and monotremes (Fig. 2.3).

2.5. DISCUSSION

We have found that marsupials and monotremes are likely to have functional classical, alternative and lectin complement pathways, as we identified the majority of the components involved in each of these complement pathways and found them to be comparable to humans, and other model eutherians. We have provided results from human/non-eutherian alignments of both nucleotide and protein sequences for genes positively identified (46 in the gray short-tailed opossum, 27 in the tammar wallaby, 44 in the Tasmanian devil, 47 in the koala genome and 40 in the platypus). With the exception of four genes – C1qL2 (opossum and wallaby), C1qTNF5 (opossum) and C9 (opossum, wallaby, devil and platypus) – we identified nucleotide sequences that had nucleotide identities ≥70%, but when the nucleotide sequences were translated into protein sequences (using BLAT), the alignments had low identity ratios or high e-values (>0) and therefore were not considered identified. Conversely, the protein sequence of Tasmanian devil C4BPB was translated to nucleotide sequence (using ExPASy) and this alignment also had a low identity ratio and high e-value.

Limitations of this study include the quality of the genomes or potential gaps in the genomes. For example, the tammar wallaby genome has not been sequenced to the same level as the gray-short tailed opossum, platypus and Tasmanian devil genomes and requires more intensive research as the lower coverage of the genome indicates that the genes may be fragmented during assembly or unannotated (Heider *et al.* 2011; Renfree *et al.* 2011). In addition, regions of the tammar wallaby genome were identified and arranged using the gray short-tailed opossum and to a certain extent the platypus genome sequence as a guide (Renfree *et al.* 2011). In other cases it is possible that the degree of divergence from the human and opossum orthologues may have resulted in a lack of sequence identification. However, the genes may be absent from all the non-eutherian mammals but this will require further investigation.

2.5.1. THE COMPLEMENT COMPONENT C3

The phylogenetic tree constructed using C3 sequences from eutherian and non-eutherian mammals indicates that C3 has diverged in all three mammal groups; eutherians, marsupials and monotremes (Fig. 3), suggesting C3 sequence is specific to the different mammal classes. EGF is a common motif that is found in several complement components including C3, which is not surprising as EGFs are highly conserved sequences in complement components (Reid and Day 1989). EGF-like domains contain a potential β-hydroxylation site (Thielens *et al.* 1990) and have a role in calcium-binding (Stenflo *et al.* 2000), which is essential for the function of the calcium-dependent classical (Zhang *et al.* 1999) and lectin complement pathways (Takahashi *et al.* 1999). EGF also plays a role in the alternative pathway by regulating CFB synthesis in fibroblasts (Circolo *et al.* 1990). Other motifs identified in C3 were vWFC and anaphylatoxin. vWFC has chordin-like cysteine-rich repeats and is involved in maintaining homeostasis (Hunt and Barker 1987) and anaphylatoxin is required for the elimination of pathogens through a local inflammatory response (Sontheimer *et al.* 2005; Klos *et al.* 2009), which has been documented in the gray short-tailed opossum when exposed to ultraviolet radiation (Kusewitt *et al.* 1991).

2.5.2. CLASSICAL COMPLEMENT PATHWAY

In mammals studied to date, the classical complement pathway is activated when antigen-antibody complexes or pathogens bind to complement component 1, q subcomponent (C1q) (Fig. 2.1). Antibodies, immunoglobulin M (IgM) and immunoglobulin G (IgG), are responsible for the activation of the classical pathway because their fragment crystallisable (Fc) region is able to attach to the multivalent C1q complex (Kishore and Reid 1999; Kishore and Reid 2000; Leslie and Nielsen 2004), through specific constant domains (Prater *et al.*

1991; Taylor *et al.* 1998). C1q not only activates the classical complement pathway by binding to antibodies mentioned, but also works with TNFs to eliminate pathogens (Kishore *et al.* 2004).

Most of the C1q components (C1qA-C, C1qBP, C1qL2, C1qL4, C1qTNF1-9) were identified in non-eutherian mammals with all C1q components identified in Tasmanian devil and koala, suggesting a functional classical complement system in non-eutherian mammals. Gene sequences for C1qTNF1, 2, 3, 5, 6 and 7 were identified in all the non-eutherian mammals. The results are not surprising as TNFs have previously been cloned and identified in marsupials (Wedlock *et al.* 1996; Harrison *et al.* 1999). Sequence motif analyses of C1qA-C identified collagen domains (Supplementary Material 2.5-2.7), suggesting the structure and function of C1q in eutherian and non-eutherian mammals are similar and therefore likely to activate the classical complement pathway.

Gene sequences for both C3 and C5 that are important for the later steps of the classical complement pathway cascade (Fig. 2.1) were found in all non-eutherian mammals. In the C3 alignments conducted (Supplementary Material 2.4), conserved regions in C3 protein sequences were detected among eutherian and non-eutherian mammals, including the CR2 and CFP binding sites, suggesting functional conservation of C3 in mammals. The presence of both C3 and C5 in the non-eutherian mammals reinforces the importance of these complement components across phylogenetic boundaries.

The gray short-tailed opossum complement component 3a receptor 1 (C3aR1) had an amino acid percent identity of 55% when compared to human C3aR1 (Table 2.1). Both human and gray short-tailed opossum C3aR1 have a G-protein coupled receptor family 1 signature, which contributes to the main function of C3aR1, which is to enhance the production of anaphylatoxin (DeFranco *et al.* 2007). C3aR1 in both species also has three 7-

transmembrane receptors, as expected as C3aR1 complement components are G-protein-coupled receptors of the 7-transmembrane type (DeFranco *et al.* 2007).

2.5.3. Lectin Complement Pathway

The four important complement components involved specifically in the lectin pathway investigated here were: mannose-binding lectin protein C2 (MBL2), ficolin collagen/fibrinogen domain containing 3 Hakata antigen (FCN3) and mannan-binding lectin serine peptidases, MASP1 and MASP2. All four complement components are important for the initiation of the eutherian lectin complement pathway and their presence in all non-eutherian mammals investigated suggest similarities in all mammalian lectin complement pathways. FCN3 was identified in the gray short-tailed opossum and tammar wallaby, while MASP1 and MASP2 were identified in all non-eutherian mammals examined here (MASP1 and MASP2 protein sequence alignments are in Supplementary Material 2.8 and 2.9). MBL2 was identified in the gray short-tailed opossum and koala.

2.5.4. Alternative Complement Pathway

In the alternative pathway, C3 is constantly hydrolysed, forming C3b, which binds to foreign surfaces, resulting in the binding of complement factor B (CFB) to C3b (called C3bB; Sarma and Ward 2011). CFB has three CCP domains followed by a vWF domain and a serine protease (Edwards and Perkins 1995; Edwards and Perkins 1996). CFB sequences of mammals also have conserved N-linked glycosylation sites that are involved in lectin recognition (Abbott *et al.*2010; Supplementary Material 2.10). As mentioned previously, in marsupials, CFB has been detected using proteomic techniques in tammar wallaby milk from

days 97 to 172 postpartum (Joss *et al.* 2009). In the genomes that we searched, CFB is identified in the gray short-tailed opossum, Tasmanian devil and koala. We also looked further into CFD that is identified in all non-eutherian mammals (except opossum), and found that the active site of CFD (known as the catalytic triad) is conserved in non-eutherian mammals (Volanakis and Narayana 1996; Supplementary Material 2.11).

The CFP component, which aids in wound healing (Tucker 2004) has seven type 1 thrombospondin repeats (TSRs; Nolan *et al.* 2005). In the sequence alignment of CFP (Supplementary Material 2.12), we found the conserved region of type I TSR and a glycosaminoglycan (GAG) binding site. The wound-healing rate of monotremes is comparable to that of eutherians, suggesting that the function of any TSRs, if expressed in monotremes, is similar to eutherians (Watson and Graves 1988). Gray short-tailed opossum neonates younger than nine days old have demonstrated the ability to heal without macroscopic scarring and are able to re-establish a normal thickness at the wound site within seven days (Armstrong and Ferguson 1995), which suggests, but does not confirm, the potential role of TSRs in this class of mammals.

CFP was identified in all non-eutherian mammals except for the tammar wallaby. Non-eutherian CFP sequences identified in this study were found to have two common motifs, defensin and integrin β, both of which have previously been identified in the gray short-tailed opossum (Lynn and Bradley 2007; Holmes and Rout 2011). Defensin is essential for the innate immune system because of its antimicrobial properties in eutherian and non-eutherian mammals (Lynn and Bradley 2007) and specifically target Gram-negative bacteria (Borregaard *et al.* 2000).

Integrin with β-chains, including integrin β, are usually in cysteine-rich domains and play various roles in the immune system (Holmes and Rout 2011). β_2 integrins are required for the expression of complement-dependent and oxygen radical-mediated injury of the lung

and dermal vasculature in neutrophils and are an important component for inflammation and immune clearance (Ueda *et al.* 1994). The presence of defensin and integrin β genes suggests the importance of CFP function for the immune system of mammals.

2.5.5. Inhibitors of Complement Pathways

In our study, at least one inhibiting complement component was identified in each of the non-eutherian mammals. Inhibitors of complement pathways aid regulation of the complement cascades by inhibiting part of the complement protein cascade. One of the main inhibitors for all three complement pathways is complement factor I (CFI). C3b and C4b complement factors that are bound to host tissues are prevented from cleaving by CFI and a co-factor protein of either CD46, CR1, complement factor H (CFH), C4-binding protein (C4BP) or C4-binding protein β-chain (C4BPB) (Scharfstein *et al.* 1978; Sarma and Ward 2011), which prevents the formation of a C3-convertase (Goldberger *et al.* 1987; Hourcade *et al.* 1992). The co-factor proteins that bind to CFI are mostly composed of Sushi domains. CFI is present in all non-eutherian mammals, except for tammar wallaby, which suggests the ability of non-eutherian mammals to regulate the formation of C3-convertase in the three main complement pathways.

CFH and C4BP, complement components that were also investigated in this study, inhibit the alternative pathway by accelerating the decay of the C3-convertase (Bao *et al.* 2010) and is a regulator of C3-convertase in the classical component pathway (Blom *et al.* 2001). CFI is present in all non-eutherian mammals except for the tammar wallaby, while at least one component of C4BP (C4BPA and C4BPB) is present in all non-eutherian mammals. In humans, CFH is genetically and structurally linked to complement factor H-related (CFHr) 1-5, but the exact biological function of CRHr is unknown. CFH is the main inhibitor of

alternative and lectin pathways in the complement system and aids in complement regulation in both fluid-phase and cellular surfaces (Rodriguez de Córdoba *et al.* 2004). CFHr1, in particular, has been found to disrupt the formation of MAC and CFHr5 is involved in the regulation of C3-convertase (Abarrategui-Garrido *et al.* 2009). CFH was identified in the gray short-tailed opossum, Tasmanian devil, koala and platypus, and at least one CFH-related protein (CFH-related 1-5) is present in all non-eutherian mammals, except for the Tasmanian devil and koala.

CR1 is able to prevent the assembly of MAC. However CR1 also up-regulates the opsonisation of pathogens as it recognises C3b on the surface of a pathogen and induces a phagocytic response when a cell is co-stimulated by C5a binding to the C5a receptor (Kelley *et al.* 2010) and is also involved in the cleaving of C2 and C4 in the classical complement pathway (Arlaud and Thielens 1993). CR1 was found in the Tasmanian devil and platypus.

Another main inhibitor that regulates all three complement pathways is CD59, which interferes with the formation of the MAC on foreign cell surfaces (Huang *et al.* 2006). Surprisingly, CD59 was not found in any of the non-eutherian mammals. Its absence may be due to the limitations of the BLAST program.

Using a genome survey approach, we successfully identified complement genes in four marsupials and a monotreme. By comparing the complement components of C1qA-C, CFB, CFD, CFP, MASP1-2 and C3, we found that they are largely similar in nucleotide sequence and contain motifs that suggest that translated proteins will likely be functionally equivalent to homologues in humans and other eutherian species. Despite some limitations to the survey that include sequencing errors or software restrictions, we identified a significant number of gene transcripts that together, comprise a complex complement system. Complement sequences that are highly conserved are likely to yield the same search results in non-

eutherians. For example, human C4A and C4B nucleotide sequences have a conserved sequence with 5413/5426 (99%) nucleotide identity and therefore resulted in the BLASTN search identifying only one nucleotide sequence in the koala genome for both sequences. The similarities between C4A and C4B sequences indicate that the differences between the two genes require further investigation.

The complement system plays an important role in the immune system of eutherians, and is therefore likely to have a comparable role in the immune system of non-eutherian mammals. This study provides the platform for future studies that want to further analyse complement proteins in various marsupials and monotremes, and specifically to study the immune mechanisms available to marsupial pouch young.

2.6. CHAPTER 2 REFERENCES

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2.7 Chapter 2 Supplementary Materials

Additional Supporting Information may be found in the appendix:

Supplementary Material 2.1 Number of nucleotide/amino acids, best hit (e-value) and percentage of nucleotide and amino acid identity of respective complement components to their human homologues.

Supplementary Material 2.2 The database origins of human and non-eutherian complement components.

Supplementary Material 2.3 The number one represents the presence of a motif in the sequence of the human and non-eutherian mammals. EGF = epidermal growth factor; Integrin β = integrins beta chain cysteine-rich domain signature; vWFC = von Willebrand factor C. Grey areas indicate that the species' genomic sequence was not found in the Genbank and Ensembl database. Species names were abbreviated using the first letter from the genus and the first two letters of the species name followed by the gene name.

Supplementary Material 2.4 Alignment of C3 amino acid sequences. As C3 is a central component of the complement system, it has many binding sites that will continue the complement system after it has been activated through the classical, alternative and lectin pathway. Underlined is the signal peptide from the B-chain of the C3. CR1, CR2, CR3, H and B binding site is the first box (black). H, CR2 binding site is the second sequence box (red). CFP binding site is the third sequence box (blue; adapted from Sahu and Lambris (2001). Highlighted area shows 70% similarity between sequences. These genes are abbreviated using the first letter from the genus and the first two letters of the species name followed by the gene name. Sequences used for this alignment: *Homo sapiens* (GENBANK: AAA85332.1), *Monodelphis domestica* (GENBANK: XP_003340773.1), *Macropus eugenii*

(GENBANK: AAW69835.1), *Ornithorhynchus anatinus* (ENSEMBL: ENSOANP00000009738), *Isoodon macrourus* (GENBANK: ABC84125.1; partial), *Mus musculus* (GENBANK: AAH43338.1), *Sus scrofa* (GENBANK: AAG40565.1), *Bos taurus* (GENBANK: AAI12453.1).

Supplementary Material 2.5 Alignment of C1qA amino acid sequences. The (Gly-Xaa-Yaa)$_n$ representing the collagen region is boxed. Highlighted area shows 70% similarity between sequences. These genes are abbreviated using the first letter from the genus and the first two letters of the species name followed by the gene name. Sequences used for this alignment: *Homo sapiens* (GENBANK: NM_015991.2), *Monodelphis domestica* (GENBANK: XP_001376435.1), *Sarcophilus harrisii* (GENBANK: XP_003764946.1) *Ornithorhynchus anatinus* (ENSEMBL: ENSOANP00000024235), *Sus scrofa* (GENBANK: AAR20892.1), *Mus musculus* (GENBANK: EDL29926.1), *Canis lupus familiaris* (GENBANK: XP_535367.1), *Gallus gallus* (GENBANK: XP_004947579.1).

Supplementary Material 2.6 Alignment of C1qB amino acid sequences. The (Gly-Xaa-Yaa)$_n$ representing the collagen region is boxed. Highlighted area shows 70% similarity between sequences. These genes are abbreviated using the first letter from the genus and the first two letters of the species name followed by the gene name. Sequences used for this alignment: *Homo sapiens* (GENBANK: AAH08983.1), *Monodelphis domestica* (GENBANK: XP_001379091.1), *Sarcophilus harrisii* (GENBANK: XP_003775335.1), *Mus musculus* (GENBANK: AAH67001.1), *Gallus gallus* (GENBANK: XP_425756.2), *Bos taurus* (GENBANK: AAI12491.1), *Canis lupus familiaris* (GENBANK: XP_544507.2).

Supplementary Material 2.7 Alignment of C1qC amino acid sequences. The (Gly-Xaa-Yaa)$_n$ representing the collagen region is boxed. Highlighted area shows 70% similarity between sequences. These genes are abbreviated using the first letter from the genus and the

first two letters of the species name followed by the gene name. Sequences used for this alignment: *Homo sapiens* (GENBANK: AAH09016.1), *Monodelphis domestica* (GENBANK: XP_001376425.2), *Macropus eugenii* (ENSEMBL: ENSMEUT00000008332), *Sarcophilus harisii* (GENBANK: XP_003764925.1), *Ornithorhynchus anatinus* (GENBANK: XP_001518772.2), *Bos taurus* (GENBANK: AAI14894.1; partial), *Canis lupus familiaris* (GENBANK: XP_003433793.1), *Sus scrofa* (GENBANK: AAR20893.1; partial), *Mus musculus* (GENBANK: AAH43945.1).

Supplementary Material 2.8 Alignment of MASP1 amino acid sequences. Unknown amino acids are labelled X. Highlighted area shows 70% similarity between sequences. These genes are abbreviated using the first letter from the genus and the first two letters of the species name followed by the gene name. Sequences used for this alignment: *Homo sapiens* (GENBANK: AAI06947.1), *Monodelphis domestica* (GENBANK: XP_007502502.1), *Macropus eugenii* (ENSEMBL: ENSMEUT00000000085), *Sarcophilus harrisii* (GENBANK: XP_003766301.1), *Ornithorhynchus anatinus* (GENBANK: XP_007667766.1), *Mus musculus* (GENBANK: AAI31639.1), *Rattus norvegicus* (GENBANK: AAH85685.1).

Supplementary Material 2.9 Alignment of MASP2 amino acid sequences. Highlighted area shows 70% similarity between sequences. These genes are abbreviated using the first letter from the genus and the first two letters of the species name followed by the gene name. Sequences used in this alignment: *Homo sapiens* (GENBANK: EAW71674.1), *Monodelphis domestica* (ENSEMBL: ENSMODT00000014293.3), *Macropus eugenii* (ENSEMBL: ENSMEUT00000003109), *Sarcophilus harrisii* (GENBANK: XP_003765058.1), *Ornithorhynchus anatinus* (GENBANK: XP_001510548.3), *Mus musculus* (GENBANK: AAH13893.1), *Rattus norvegicus* (GENBANK: AAI28701.1).

Supplementary Material 2.10 Alignment of CFB amino acid sequences. Potential N-linked glycosylation sites are boxed (Perkins *et al.* 1994). Highlighted area shows 70% similarity between sequences. These genes are abbreviated using the first letter from the genus and the first two letters of the species name followed by the gene name. Sequences used for this alignment: *Homo sapiens* (GENBANK: EAX03550.1), *Monodelphis domestica* (GENBANK: XP_007483634.1), *Sarcophilus harrisii* (GENBANK: XP_003768987.1), *Rattus norvegicus* (GENBANK: AAH87089.1), *Mus musculus* (GENBANK: XP_011236443.1), *Ovis aries* (GENBANK: ABR01165.1), *Sus scrofa* (GENBANK: ABX82825.1).

Supplementary Material 2.11 Alignment of CFD amino acid sequences. The catalytic triad of CFD (Asp^{102}, His^{57} and Ser^{195}) is labelled with arrows (Volanakis and Narayana 1996). Highlighted area shows 70% similarity between sequences. These genes are abbreviated using the first letter from the genus and the first two letters of the species name followed by the gene name. Sequences used for this alignment: *Homo sapiens* (GENBANK: AAH57807.1), *Macropus eugenii* (ENSEMBL: ENSMEUT00000011987), *Sarcophilus harrisii* (GENBANK: XP_003760881.1), *Ornithorhynchus anatinus* (GENBANK: XP_003429544.2), *Mus musculus* (GENBANK: AAI45413.1), *Bos taurus* (GENBANK: AAI02480.1).

Supplementary Material 2.12 Alignment of CFP amino acid sequences. A thrombospondin repeat is underlined (Prater *et al.* 1991). GAG-binding site is boxed in red (Guo *et al.* 1992). Highlighted area shows 70% similarity between sequences. These genes are abbreviated using the first letter from the genus and the first two letters of the species name followed by the gene name. Sequences used for this alignment: *Homo sapiens* (GENBANK: AAB62886.1), *Monodelphis domestica* (GENBANK: XP_001371216.2),

Sarcophilus harrisii (GENBANK: XP_003775025.1), *Ornithorhynchus anatinus* (ENSEMBL: ENSOANT00000009001), *Mus musculus* (GENBANK: EDL00734.1), *Loxodonta africana* (GENBANK: XP_003418055.2), *Equus ferus caballus* (GENBANK: XP_001492706.1), *Oryctolagus cuniculus* (GENBANK: XP_008270776.1).

CHAPTER THREE

EVALUATION OF REFERENCE GENES FOR GENE EXPRESSION IN RED-TAILED PHASCOGALE LIVER, LUNG, SMALL INTESTINE AND SPLEEN

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3.1. CHAPTER OUTLINE AND AUTHORSHIP

Chapter 3 evaluated the stability of reference genes in four immune tissues of red-tailed phascogales; liver, lung, small intestine and spleen. The stability of reference genes is important in real-time PCR studies because targeted genes have to be normalised to two stable reference genes. This manuscript will assist future expression studies as they can use the genes identified in this study as stable reference genes. The study was conducted with the approval of the Western Sydney University's Animal Care and Ethics Committee, A9872.

The following manuscript is jointly authored, where I am the primary author and performed RNA extraction, cDNA synthesis, generating primer pairs, conducted other laboratory analysis, and wrote the manuscript. Associate Professor Julie Old and Dr. Lauren Young supervised the development of the study, provided editorial feedback on manuscript drafts and Associate Professor Julie Old acted as the corresponding author of the manuscript.

The authors of this manuscript would like to thank the editor and anonymous reviewers who provided critical feedback on the manuscript prior to being accepted and published by the journal. This chapter is a published journal article and should be cited as:

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3.2. INTRODUCTION

Fluorescence-based quantitative real-time polymerase chain reaction (qPCR) has the capacity to monitor the amplification of cDNA during thermocycling, starting with the use of ethidium bromide for the detection of fluorescence (Higuchi et al. 1992). Over the past 13 years, qPCR has developed into the most accurate and sensitive method to study gene expression with low concentrations of mRNA (Bustin, 2000; Schmittgen & Livak, 2008). When studying the expression of a target gene, it is important to have a stable reference gene for the normalisation of gene expression (Pierzchała et al., 2011). At present, there has not been a study on the stability of reference genes in a marsupial. In most cases, reference genes found stable in other mammalian groups (particularly eutherians) have been used in studies without considering the stability of the reference genes, which may differ between mammal groups and tissues/organs.

The aim of this study was to identify stable reference genes across a range of tissues of a marsupial species – the Red-tailed phascogale (*Phascogale calura*). Red-tailed phascogales are a model species as they are small and relatively easy to maintain in captivity (Foster et al., 2006; Russell, 1982; Stannard et al., 2013). In the wild, the red-tailed phascogale inhabits a small corner of the south west of western Australia (Bradley, Foster & Taggart, 2008) and are distinguished from other small marsupials by their long brush-like hairs on the end of their red tail (Kennedy & Williams, 1990).

This study evaluates the expression stability of five different reference genes in four different tissues associated with immunity in the red-tailed phascogale; liver, lung, small intestine and spleen in two different age groups. Optimal reference genes should be considered stable and expressed at constant levels in various tissues and age groups. Liver and lung tissues were primarily chosen because they contain large populations of macrophages and are in regular contact with pathogens (Laskin et al., 2001). In addition,

unlike eutherian livers that cease haematopoiesis prior to birth, the marsupial liver is the main site of haematopoiesis during early postnatal life (reviewed by Borthwick, Young & Old, 2014; Old & Deane, 2000).

Lung and small intestine were chosen as representative mucosal-associated lymphoid tissue (MALT). The MALT in lungs is responsible for protection of the respiratory system (Mak, Saunders & Jett, 2013), whilst the small intestine is an important gut-associated lymphoid tissue (GALT). In marsupials, GALT can be localised in Peyer's patches or follicular aggregations, or appear as scattered cells distributed throughout the gut (Old & Deane 2000). The last of the tissues chosen for use in this study was spleen. The spleen is an important haematopoietic site and actively involved in the adaptive immune response (reviewed by Borthwick, Young & Old, 2014; Old & Deane 2000). The four red-tailed phascogale tissues (liver, lung, small intestine and spleen) were therefore chosen based on their immunological capacity and function. The expression stability of five reference genes in these tissues was investigated using the geNorm application in the qBase^{PLUS} software.

3.3. Materials And Methods

3.3.1. Animal and Tissue Collection

Ten male red-tailed phascogales from two age groups (juveniles: 3.5 – 5 months, and adults: 1.2 – 1.5 years) were utilised in this study. Tissue samples were opportunistically obtained from a the Small Native Mammal Teaching and Research Facility, a captive colony housed at the Western Sydney University (WSU) (Richmond, NSW) as per standard operating procedures approved by the UWS Animal Ethics Committee during population maintenance. Samples of liver, lung, small intestine and spleen were dissected, and immediately stored at -80°C until total RNA extraction.

3.3.2. RNA EXTRACTION AND CDNA SYNTHESIS

Total RNA was extracted using the SV Total RNA Isolation System (Promega, Wisconsin, USA) according to the manufacturer's protocol. The quantity and quality of total RNA were estimated using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, Delaware, USA). One µg of total RNA was reverse transcribed with SuperScript® III First-Strand Synthesis SuperMix (Invitrogen, California, USA) according to the manufacturer's protocol. The final cDNA products were aliquoted and stored at -20°C until use.

3.3.3. PRIMERS AND REAL-TIME PCR

We selected five reference genes used previously in marsupial gene expression studies (Maher et al. 2014; Markey et al. 2007; Yu et al. 2006): glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), β-actin (*ACTB*), *18S* rRNA (*18S*), *28S* rRNA (*28S*) and ribosomal protein L13a (*RPL13A*). Real-time PCR primers, *GAPDH* and *ACTB*, were designed using consensus sequences based on marsupial species obtained from GenBank (Tasmanian devil *Sarcophilus harrisii*) *GAPDH*: XM_012550750.1; gray short-tailed opossum (*Monodelphis domestica*) *GAPDH*: XM_007503905.1; Tasmanian devil *ACTB*: XM_003761554.2). Primers for *18S* and *28S* were obtained from Daly et al. (2009), and *RPL13A* from Siddle et al. (2013). Specific information for each primer is listed in Table 3.1

Table 3.1 Candidate reference genes evaluated in this study.

Gene symbol	Gene name	Oligo sequence (5' → 3')	Amplicon size (bp)	Annealing temp. (°C)
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Forward: CAGGCGGAGTAGACATTG	90	60
		Reverse: CCTTGAACTTGCCATGGG		
ACTB	β-Actin	Forward: CTCTTCCAGCCATCTTTCTT	100	60
		Reverse: GACATCCGTAAGGATCTGTA		
28S	*28S* Ribosomal RNA	Forward: CGATGTCGGCTCTTCCTATC	203	60
		Reverse: TCCTCAGCCAAGCACATACA*		
		Daly et al. (2009).		
		*Reverse primer was modified according to marsupial sequences.		
18S	*18S* Ribosomal RNA	Forward: CCAACACGGGAAACCTCA	121	60
		Reverse: AACCAGAAATCGCTCCAC		
		Daly *et al* (Daly et al. 2009).		
RPL13A	Ribosomal protein L13a	Forward: CCCCACAAGACCAAGCGAGGC	145	60
		Reverse: ACAGCCTGGTATTTCCAGCCAACC		
		Siddle (Siddle et al. 2013).		

PCR amplification was performed using a Rotor Gene Q (Qiagen, Hilden, Germany) and the Rotor-Gene SYBR® Green PCR kit (Qiagen). A PCR mix (25 µL) was prepared: 7.5 µL water, 2.5 µL primers (forward and reverse; 10 µM), 1 µL (100 ng) cDNA, and 12.5 µL of Rotor-Gene SYBR Green PCR Master Mix. The following amplification program was used: 5 min denaturation at 95°C, 35 cycles of amplification with 5 s at 95°C (denaturation), 10 s at 60°C (annealing), and 15 s at 72°C (elongation). Annealing temperatures were optimised according to individual genes and primers. A melting step was performed to confirm a single gene-specific peak by a stepwise temperature increase ranging from 60°C to 95°C at ramp rate 1°C/s with continuous monitoring of fluorescence. Further analysis of amplicon specificity and size were also evaluated running qPCR products in a standard 2% agarose gel electrophoresis. Standard curves were made to calculate the amplification efficiency during real-time PCR using five-fold serial dilutions of cDNA for each tissue and each reference gene in one adult male red-tailed phascogale. The quantification cycle (C_q) was automatically determined for each reaction by the Rotor Gene Software (v. 1.7.94).

3.3.4. DATA ANALYSIS

Gene expression variation was calculated for individual reference genes based on cycle threshold (C_q) values and real-time PCR efficiencies (*E*). The real-time *E* value was calculated from the given slopes in the qBase^{PLUS} software (Hellemans et al. 2007) according to the equation: ($E = 10(-1/slope)-1$). Only C_q values <40 were used for calculation of *E* values. C_q and *E* values were then analysed in geNorm on the qBase^{PLUS} software, which ranks the reference genes based on the *M* values

(reference genes with the lowest *M* value is considered most stable). A one-way ANOVA was also performed on C_q values obtained from the expression in juveniles and adults of the four reference genes.

3.4. RESULTS

Five reference genes were amplified in four tissues, and all real-time PCR assays produced a single peak on the melting curve. The linear correlation coefficient (R^2) of all genes ranged from 0.97-1.00. C_q values for all genes in all samples were within 10.41-33.19 cycles, and were covered by the range of their respective standard curves. *E* value of reference genes, mean C_q values and range of C_q values for each tissue are depicted in Table 3.2. All reference genes in all tissues had a C_q value below 29, indicating an abundance of target nucleic acid in cDNA samples (Fig. 2.1). C_q values observed for all reference genes in this study were insignificant in juvenile and adult tissues ($p \geq 0.05$).

C_q and *E* values were used in qBase^{PLUS} to calculate expression stability (*M* value). *M* values are used to rank reference genes based on the stability, depending on the type of tissue (Fig. 3.2). *M* values below 1.5 indicate a stable expression. According to the results obtained, the most stable genes in liver are *ACTB* and *GAPDH*, lungs *RPL13A* and *GAPDH*, small intestine *ACTB* and *18S*, and spleen *ACTB* and *18S*.

Table 3.2 Cycle threshold (C_q) and reaction efficiency (E) values for individual genes in examined tissues of juveniles and adults.

	GAPDH	***ACTB***	***18S***	***28S***	***RPL13A***
Liver					
Mean C_q	26.242	27.226	14.526	15.382	27.907
Range of C_q	22.55-29.98	22.07-30.09	9.66-18.48	9.83-21.28	22.27-33.19
E (out of 2)	1.855 ± 0.047	1.716 ± 0.08	1.953 ± 0.035	1.89 ± 0.011	1.851 ± 0.029
Lung					
Mean C_q	24.6	21.746	12.995	14.476	21.924
Range of C_q	22.74-28.06	19.17-24.84	10.41-16.64	10.8-20.14	19.72-24.43
E (out of 2)	1.94 ± 0.058	1.732 ± 0.04	1.912 ± 0.026	1.909 ± 0.035	1.763 ± 0.032
Small Intestine					
Mean C_q	23.981	23.849	13.867	15.04	22.618
Range of C_q	21.75-25.50	20.67-27.47	11.56-17.67	12.32-17.50	18.89-28.53
E (out of 2)	1.919 ± 0.043	1.753 ± 0.058	1.833 ± 0.033	1.703 ± 0.013	1.811 ± 0.067
Spleen					
Mean C_q	23.541	23.566	15.361	16.673	20.661
Range of C_q	21.24-26.89	21.64-26.56	13.46-17.81	14.8-17.59	18.92-23.40
E (out of 2)	1.879 ± 0.033	1.693 ± 0.043	1.768 ± 0.036	1.978 ± 0.029	1.801 ± 0.028

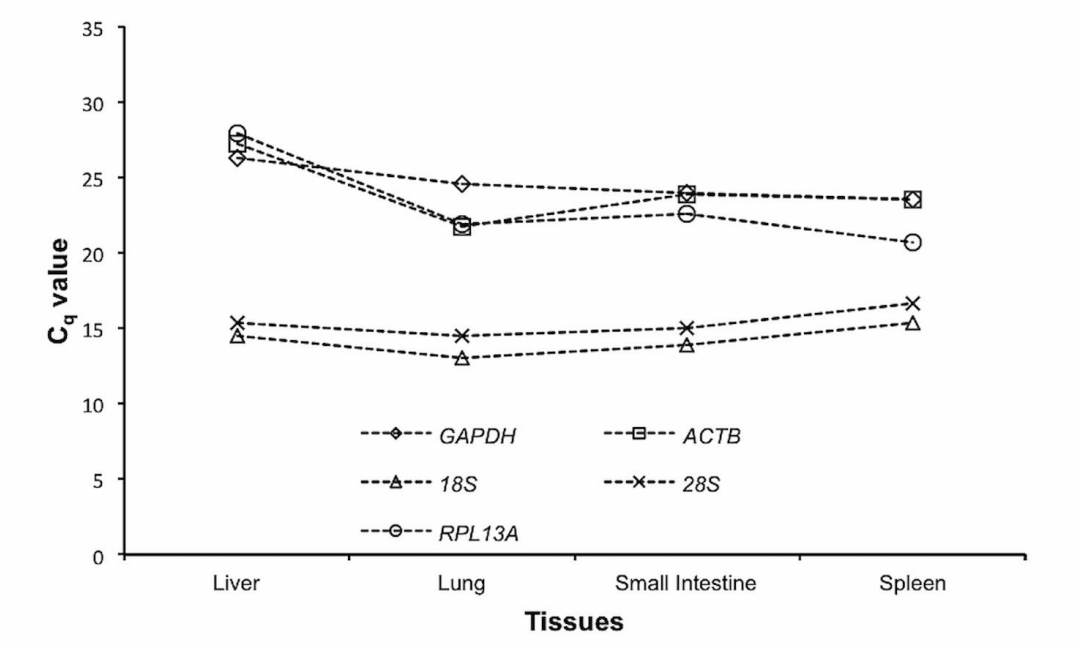

Figure 3.1 Average RNA transcription levels of putative reference genes presented as absolute Cq values. The reference genes are: Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), β-Actin (*ACTB*), 18S rRNA (*18S*), 28S rRNA (*28S*) and ribosomal protein L13a (*RPL13A*) in liver, lung, small intestine and spleen tissue samples.

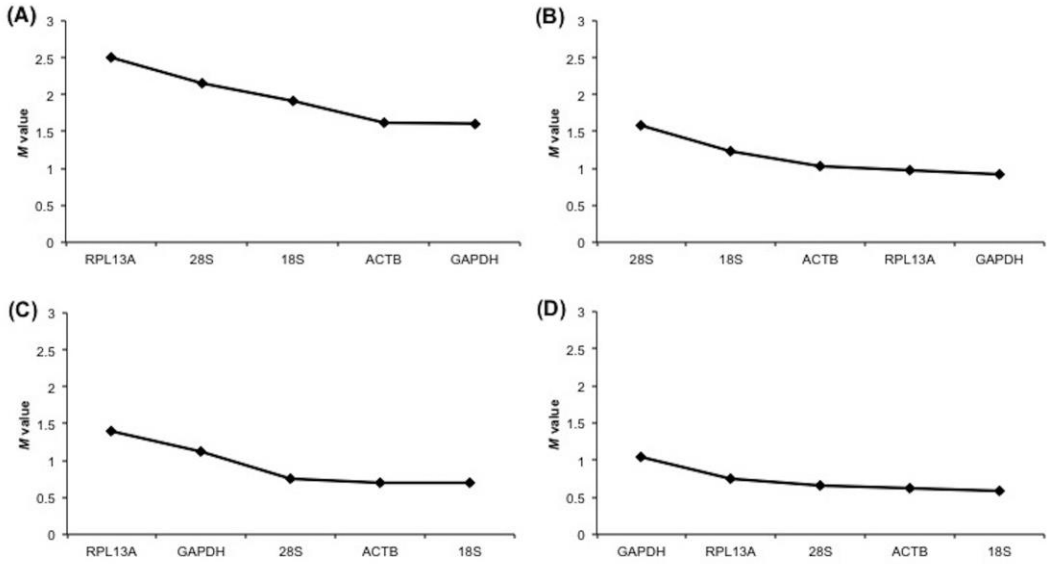

Figure 3.2 Overall expression stability values (*M*) of reference genes in tissues of different ages. Gene expression stability of reference genes in juvenile and adult red-tailed phascogale tissues analysed by geNorm application (qBase^{PLUS}) for **(A)** liver, **(B)** lung, **(C)** small intestine, and **(D)** spleen tissue. The reference genes are: Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), β-Actin (*ACTB*), 18S rRNA (*18S*), 28S rRNA (*28S*) and ribosomal protein L13a (*RPL13A)*. Genes with the lowest *M* values have the most stable expression.

As per Nygard et al. (2007), *M* values lower than 1.5 were considered stable reference genes. Upon analysis using the geNorm application, *M* values for *ACTB* and *GAPDH* in all liver tissue samples were below 1.5, indicating their stability for use in the developing red-tailed phascogale studies. In addition, all reference genes except *28S* were stable for lung tissue, and all reference genes were stable in both small intestine and spleen tissue samples (Fig. 3.2; Table 3.3).

Table 3.3 Control genes ranked in order of their expression stability*. Reference genes with *M* values below 1.5 are considered as suitable reference genes for a particular immune tissue and are bold.

Liver	**Lung**	**Small Intestine**	**Spleen**
RPL13A	*28S*	***RPL13A***	***GAPDH***
28S	***18S***	***GAPDH***	***RPL13A***
18S	***ACTB***	***28S***	***28S***
ACTB	***RPL13A***	***ACTB***	***ACTB***
GAPDH	***GAPDH***	***18S***	***18S***

*Increasing expression stability from top to bottom.

When the two age groups were analysed individually, all reference genes had *M* values below 1.5 for adult small intestine, juvenile and adult spleen tissue samples. The two most stable reference genes differed in juvenile and adult liver tissues: *M* values for *18S* and *28S* for adult liver samples were considered stable, whereas *ACTB* and *GAPDH* were stable in juvenile liver samples. All reference genes except *28S* were considered stable in both juvenile and adult lung samples, and all reference genes except *RPL13A* were considered stable in juvenile small intestine tissue samples (Fig. 3.3).

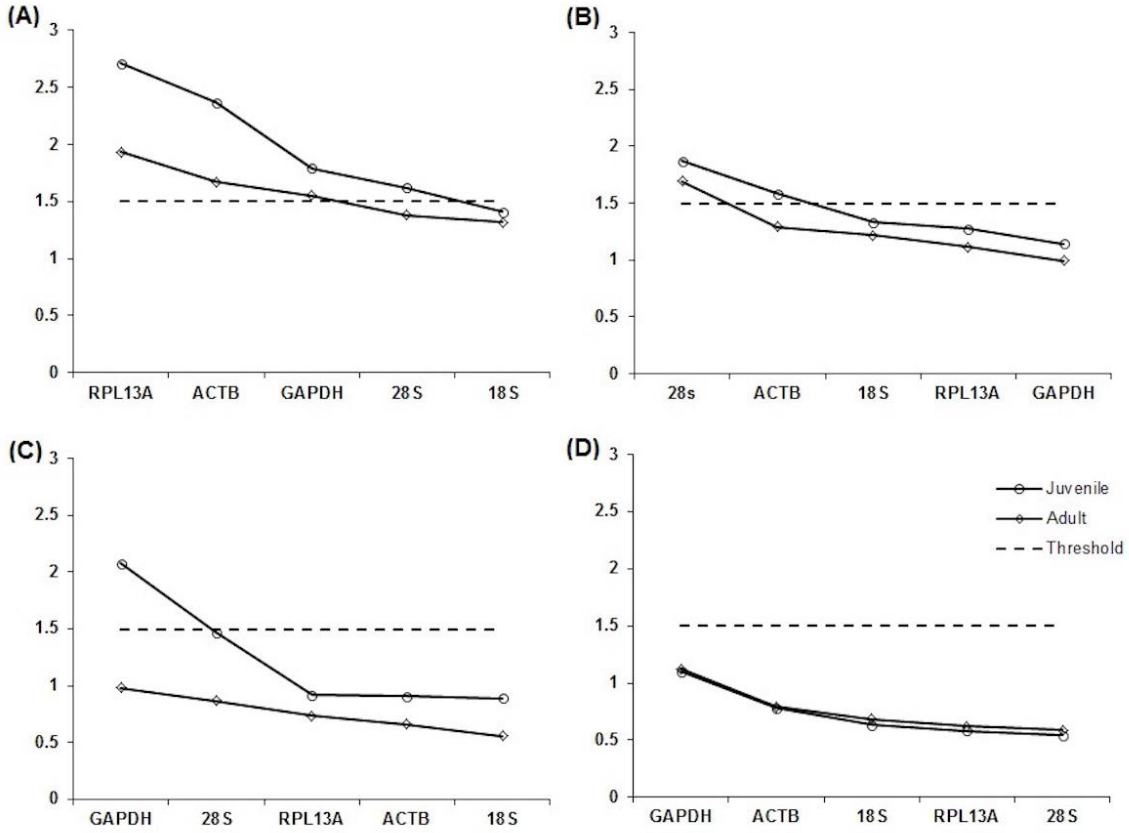

Figure 3.3 Expression stability values (*M*) of reference genes in tissues of juvenile and adults. Gene expression stability of reference genes in juvenile and adult red-tailed phascogale tissues analysed by geNorm application (qBase^{PLUS}) for **(A)** liver, **(B)** lung, **(C)** small intestine, and **(D)** spleen tissue, analysed by geNorm application (qBase^{PLUS}). The reference genes are: Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), β-Actin (*ACTB*), 18S rRNA (*18S*), 28S rRNA (*28S*) and ribosomal protein L13a (*RPL13A)*. Genes with the lowest *M* values have the most stable expression.

3.5. DISCUSSION

The ideal reference gene should constantly be transcribed in the type of tissue being examined (Nygard et al., 2007). Studies looking at the expression of reference genes in multiple tissues have however demonstrated that the regulation of reference genes are tissue specific (Lisowski et al., 2008). In this study, we provided a detailed analysis of the stability and expression levels of five different reference genes previously used in marsupial expression studies (Daly et al., 2009; Maher et al., 2014; Menzies et al., 2009; Siddle et al., 2013), in four different red-tailed phascogale tissues. We found at least two stable reference

genes with *M* values >1.5 for liver, lung, small intestine and spleen, and that all reference genes were suitable for expression studies of red-tailed phascogale small intestine and spleen tissues, which is useful as the normalisation of gene expression requires at least two reference genes (Bustin, 2000). The average C_q values for all reference genes in all tissues were below 29 cycles, indicating strong positive reactions of the target tissue to the reference genes (Fig. 3.1).

Not unexpectedly, the results of this study showed that expression stability differs between different tissues, and confirm that reference genes are expressed in every cell but are regulated differently in different tissues (Lisowski et al., 2008). *GAPDH* is one of the most commonly used reference genes for normalisation in mammalian tissues. Studies have found *GAPDH* expression to be unstable as its expression differs, for example, according to age and sex of individuals (Barber et al., 2005). Therefore, it was not surprising that there were significant differences in *GAPDH* expression across tissues used in this study; *GAPDH* expression was most stable for liver and lung, and least stable for spleen tissues (Fig. 3.2; Table 3.3). When separated into two age groups, *GAPDH* expression had the highest stability in juvenile liver tissues, and a combination of the two ages. *GAPDH*, along with *ACTB*, were stable for all tissue samples, except for adult liver tissues.

ACTB is another reference gene commonly used for normalisation in mammalian tissues (Menzies et al., 2012; Nygard et al., 2007). Foss, Baarsch & Murtaugh (1998) found that the levels of *ACTB* were more variable than *GAPDH*, and that high levels of *ACTB* were found in the porcine small intestine and spleen. However, we found that *ACTB* was more stable than *GAPDH* in small intestine and spleen tissue. In particular, juvenile small intestine and spleen tissues had the highest *ACTB* stability. Selvey et al. (2001) found that *ACTB* is an unstable reference gene in mouse sarcoma cells (matrigel), and found *18S* to be more stable.

When combining both age groups, our study agrees with Selvey et al. (2001), as *ACTB* was suitable for normalisation in small intestine and spleen tissue, however it was still less stable than *18S* in the same tissues. The same results were also observed using adult small intestine and spleen tissue samples.

Both *18S* and *28S* are often recommended as reference genes because ribosomal RNA has little variation among mammalian tissues and is often used as a successful internal standard (Goidin et al., 2001; Thellin et al., 1999). In addition, *18S* and *28S* were found to work effectively for normalisation in marsupial tissues (Janke et al., 2002; Maher et al., 2014). In this study, *18S* and *28S* were suitable for normalisation in small intestine and spleen tissue, with *18S* being the most stable. *18S* and *28S* were also the only two stable reference genes for adult liver tissue.

The last reference gene used in this study was *RPL13A*, a gene that encodes a protein in the 60S subunit of ribosomes (Vandesompele et al., 2002). Szabo et al. (2004) found *RPL13A* to be the best universal reference gene in various human tissues, including lung and small intestine. In addition, *RPL13A* had been selected by Ahn et al. (2008) as one of two ideal reference genes in rhesus macaques (*Macaca mulatta*), which included the comparison of eight reference genes in six tissues, including liver and lung. In this study, *RPL13A* was found to be least stable (highest *M* value) in liver and small intestine tissue, but suitable for normalisation in lung, small intestine and spleen tissue when both ages are combined. When divided into different age groups, *RPL13A* was stable for both age groups for lung and spleen tissue and adult small intestine, showing its suitability for normalisation in most tissue samples, with the exception of liver.

The findings of this study confirmed previous research that demonstrated tissue specific regulation of some reference genes in eutherian mammals (Lisowski et al., 2008; Nygard et

al., 2007; Uddin et al., 2011) also apply to a marsupial. Pierzchala et al. (2011) and Uddin et al. (2011) did not identify any of the reference genes used in this study as a stable reference gene in porcine liver, which shows that the regulation of certain reference genes may be different in marsupials. This study also demonstrated the stability of reference genes in some, but not all, marsupial tissues that were tested. This will aid in the selection of reference genes for normalisation in future expression studies in marsupials, particularly where studies of immune-related whole tissue preparations are performed. As in eutherian mammals, marsupial expression studies are increasing because of the ability of qPCR to detect and quantify nucleic acids (Bustin, 2000). For example, expression studies have been conducted in koala (*Phascolarctos cinereus*) to test the up- or down-regulation of specific immune genes in stimulated cells (Maher et al., 2014), and whether viral RNA levels increased or decreased in association with age (Tarlinton et al., 2005). Expression studies are also useful for comparing gene expression in eutherians and marsupials (Hübler et al., 2013).

3.6. CONCLUSIONS

We have successfully found stable reference genes in lung, small intestine and spleen tissue preparations from a dasyurid marsupial. It is possible to apply this study to whole tissue gene expression studies, especially when it is associated with immunity. While gene expression may occur at the single-cell level, whole tissue studies show the mean expression of several cell types available in the tissue (Kahlem et al., 2004), which is relevant especially when more than one type of cell relates to immunity in an immune system study. Future studies that focus on isolated cell preparations from these tissues will shed further light on reference gene expression and whether or not whole tissue preparations can be directly compared with cell culture studies. Results from the present study enable recommendations

on reference genes suitable for use in various marsupial tissues and for normalisation in gene expression experiments in developing marsupials.

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CHAPTER FOUR

THE EXPRESSION OF SPECIFIC COMPLEMENT PROTEINS IN DEVELOPING RED-TAILED PHASCOGALES (*PHASCOGALE CALURA*)

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4.1. Chapter Outline And Authorship

Chapter 4 demonstrates the expression of complement proteins specific to one of three complement pathways; classical, alternative and lectin complement pathways. The study was conducted with the approval of the Western Sydney University's Animal Care and Ethics Committee, A9872 and A9694.

The following manuscript is jointly authored, where I am the primary author and performed RNA extractions, cDNA synthesis, generated primer pairs, conducted other laboratory analysis, and wrote the manuscript. Associate Professor Julie Old and Dr. Young supervised the development of the study, provided editorial feedback on manuscript drafts. Associate Professor Julie Old acted as the corresponding author of the manuscript.

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4.2 INTRODUCTION

Previously, the marsupial immune system was considered ‘primitive’ and not on par with the eutherian immune system (Jurd, 1994). Since that time and with the release of sequence data from the gray short-tailed opossum genome, we now recognise that the marsupial immune system is just as complex as its eutherian counterparts (Ong et al., 2016). One of the differences between eutherians and marsupials is that eutherian young develop some immunological support *in utero* (Graves and Westerman, 2002), while marsupials are born immunologically immature (Old and Deane, 2000).

Almost 50 years ago, Wirtz and Westfall (1967) described a functional complement system in the Virginian opossum (*Didelphis virginiana*). Very little work followed this early study, but Koppenheffer et al. (1998) reported on complement proteins in gray short-tailed opossums (*Monodelphis domestica*) and more recently we described complement activation in red-tailed phascogales (*Phascogale calura*; Ong et al., 2015). Thus, we know that adult marsupials appear to use the complement system as part of their immune repertoire.

The complement system has many immunological functions including mediating inflammatory responses (Sarma and Ward, 2011). Therefore, there is a possibility that the complement system plays a role in various immunological functions in marsupials including inflammatory responses in semelparous males. Components involved in the complement system have been reported in vertebrates and invertebrates, suggesting its presence in a common ancestor and diversified in different speciess (Nonaka, 2001). Therefore, comparative studies of complement components, their mode of activation and their role in the protection of both young and adult animals across phylogenetically boundaries, will contribute to a more thorough understanding of the evolutionary role of the complement system.

The complement system is an important mediator for innate and adaptive immune systems (Carroll, 2004), and in eutherians, is comprised of more than 30 proteins collaborating to remove pathogens and apoptotic cells (Sarma and Ward, 2011). The three most-studied complement pathways in eutherians are the Classical, Alternative and Lectin Complement pathways, distinguished by different activation methods and the use of specific complement proteins (Moghimi et al., 2011). The three complement pathways converge at a step where complement component 3 (C3) is cleaved (Moghimi et al., 2011), hence C3 is a critical protein in complement function. The expression of the C3 gene may thus not only serve as an indicator of a functional complement system but also as an indicator for the 'immunological switch' for the expression of complement genes in developing marsupial young.

This study confirms the gene expression of C3, complement component 1, r subcomponent (C1r) and complement factor properdin (CFP) in red-tailed phascogale and their young, and report the presence of Mannan-Binding Lectin Serine Protease 2 (MASP2) RNA, which confirms the likely existence of the Lectin Complement pathway. In order to further investigate the role of complement in the early pouch life of marsupials, we quantified the expression of four key complement components: C1r, CFP, MASP2, and C3 in developing red-tailed phascogales.

Red-tailed phascogales are small arboreal, carnivorous marsupials found in Western Australia (Bradley, 1997), and are model marsupials for scientific research because they are easily kept in captivity (Stannard et al., 2013) and breed annually in July (Bradley, 1987; Foster et al., 2008). This data will help to establish the role of complement prior to the development of an adaptive immune system in young marsupials, a role that has not been previously investigated.

4.3 Materials and Methods

4.3.1 Animals And Tissue Collection

Red-tailed phascogale samples were collected opportunistically from the Small Native Mammal Teaching and Research Facility, a captive colony housed at Western Sydney University (Richmond, New South Wales, Australia). Tissue samples were collected as per standard operating procedures approved by the Western Sydney University Animal Ethics Committee (pouch young – A9694; juveniles and adults – A9872). Red-tailed phascogales are an endangered species; therefore the sample sizes were opportunistic. We managed to collect samples from 21 presumably healthy individuals (eight age groups); six age groups of pouch young (n=15), one age group of juveniles (n=3) and one age group of adults (n=3). Pouch young ages were determined using crown-rump length according to Foster et al. (2006). Bodies (without heads) of pouch young ≤18-days-post-partum were used because their small size made it very difficult to ensure only liver tissue was dissected and incorporated in the study. Liver tissue extraction was successful for male red-tailed phascogales aged 31-days (n=2), five months (n=3), and 14 months (n=3). Whole body and liver tissues were placed in RNAlater RNA Stabilisation Reagent (Qiagen, NSW, Australia) and stored in the -80°C freezer until use.

4.3.2 RNA Extraction And cDNA Synthesis

Total RNA was extracted from bodies (1-18-days old) and liver (31-day - 14-months old) using SV Total RNA Isolation System (Promega, Wisconsin, USA) as per manufacturer's instructions. RNA concentration and quality were measured using the Nanodrop 2000 Spectrophotometer (Thermo Scientific, Delaware, USA). Samples with low concentrations of RNA were re-precipitated using ethanol to obtain a higher concentration, and the quality of

the RNA was re-analysed to ensure suitability for qPCR studies. Using the Superscript III First Strand Synthesis System (Invitrogen, California, USA), cDNA was synthesised using 1 µg of RNA as per manufacturer's instructions. The quantity and quality of cDNA were also determined using the Nanodrop 2000 Spectrometer prior to storage in the -20°C freezer until use.

4.3.3 PRIMERS

Forward and reverse primers for C3, CFP and MASP2 for initial Polymerase Chain Reactions (PCRs) were designed using consensus sequences of mammals extracted from GenBank (http://www.ncbi.nlm.nih.gov/genbank) (Benson et al., 2013) and are listed in Table 4.1. These initial primers were tested on adult liver tissue using RT-PCR, and detection of PCR products was performed by size fractionation using electrophoresis on a 2% agarose gel (80 volts; 40 min). Bands were excised from the gel, ligated into a pCR™2.1-TOPO® vector (Invitrogen, California, USA) and cloned using the TOPO TA cloning kit (Invitrogen). Plasmid DNA was extracted using Purelink® Quick Plasmid Miniprep Kit (Invitrogen) and sequenced at the Australian Genome Research Facility (AGRF), Westmead, Sydney. To confirm product identity, the sequenced products were queried in Nucleotide Basic Local Alignment Search Tools (BLASTN) (Altschul et al., 1990) available on GenBank. After confirming the product identity (e-value ≤0), Rapid Amplification of cDNA Ends (RACE)-PCR primers were designed using sense and antisense primers specific for phascogale C3, CFP and MASP2, as per manufacturer's instructions. Additional primers were designed for "primer walking" as both C3 and MASP2 RACE products were too long to be sequenced in a single sequence read. Initial PCR and RACE PCR primers are listed in Table 4.1.

C3 and MASP2 real-time PCR (qPCR) forward and reverse primers were designed based on species-specific partial sequences obtained for red-tailed phascogales. As attempts for CFP qPCR primers failed, an attempt to use a forward primer designed based on partial CFP sequence obtained for red-tailed phascogales and a reverse primer using conserved sequences was made. Forward and reverse C1r qPCR primers were designed using conserved sequences derived by the construction of multiple sequence alignments. The list for GenBank sequences used in the multiple sequence alignments of C1r and CFP are: opossum (C1r; XM_007503684.1, CFP; XM_001371179.2), Tasmanian devil (C1r; XM_012550743.1; CFP: XM_012553410.1), platypus (*Ornithorhynchus anatinus*, C1r; XM_007656725.1, CFP; XM_007659217.1), human (C1r; M14058.1, CFP; NM_002621.2), mouse (CFP; NM_008823.4) and pig (*Sus scrofa*; C1r; XM_005658545.2, CFP; XM_003135053.3).

Primer sequences for reference genes GAPDH and β-Actin were obtained from Ong et al. (2016b) and are listed in Table 4.2. Primers were initially tested using adult liver red-tailed phascogale cDNA using RT-PCR. PCR amplification products were then isolated, cloned, sequenced and analysed as described previously to confirm their identity. At least one primer in each primer pair was located across exon-exon boundaries for all primer pairs except for C1r primers, where the forward and reverse primers were located on the same exon. After optimisation of qPCR primers using liver tissue, qPCR was conducted using the Rotor-Gene Q (Qiagen, Hilden, Germany). The qPCR primer sequences and amplicon sizes are listed in Table 4.2.

4.3.4 Sequence Identification of Complement Components

For the identification of C3, CFP and MASP2 red-tailed phascogale sequences, RACE PCR was performed on 3’ and 5’-RACE-ready cDNA made using SMARTer™ RACE cDNA

Amplification Kit (Clontech, Palo Alto, California) as per manufacturer's instructions. Both 3' and 5' RACE-ready cDNA were synthesised from the liver tissue from a two-year old male red-tailed phascogale. "Primer walking" was performed using the respective 3' and 5' plasmids as products, after every sequence read. Product sizes were confirmed by running RACE-PCR products on a 2% agarose gel. Products were then ligated, cloned and sequenced as described earlier. After confirming the sequences, red-tailed phascogale nucleotide sequences were then converted into amino acid (protein) sequences using ExPASy Translate Tool (http://www.expasy.ch/tools/dna.html) for multiple sequence alignments comparing C3 and MASP2 phascogale sequences to respective sequences from other mammals.

Multiple sequence alignments were performed using ClustalW (Thompson et al., 1994) along with a phylogenetic analysis using Neighbor-Joining tree building method with Jukes-Cantor genetic distance model, both of which were performed by Geneious® 6.1.6 software (Biomatters, Auckland, New Zealand). Geneious® were also used to produce rooted phylogenetic trees from C3, MASP2 and CFP peptide alignments using BLOSUM62 as the scoring matrix (Figure 4.1). Sequence motif analyses were conducted using MOTIF (GenomeNet; http://www.genome.ad.jp/) using a PROSITE pattern notation, and conserved cDNA sequence of red-tailed phascogale C3, CFP and MASP2 were compared to known human cDNA sequences for the identification of functionally relevant residues.

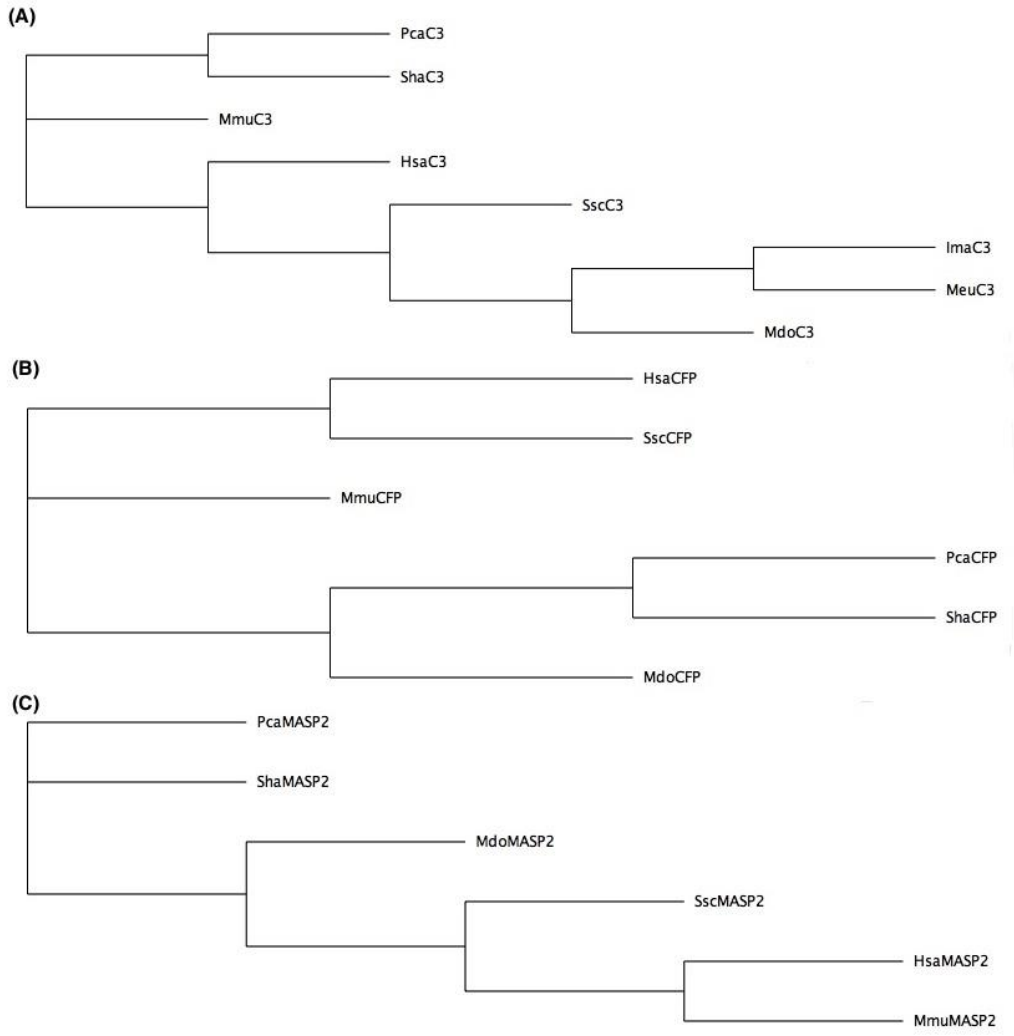

Figure 4.1 (A) The C3 phylogenetic tree was constructed using the *Phascogale calura* C3 sequence obtained from this study, along with *Homo sapiens* (AAA85332.1), *Mus musculus* (AAH43338.1), *Sus scrofa* (AAG40565.1), *Isoodon macrourus* (ABC84125.1), *Monodelphis domestica* (XP_003340773.1), *Macropus eugenii* (AAW69835.1), *Sarcophilus harrisii* (XP_012397519.1). **(B)** The CFP phylogenetic tree was constructed using the *Phascogale calura* CFP sequence obtained from this study, along with *Homo sapiens* (AAB62886.1), *Mus musculus* (EDL00734.1), *Sus scrofa* (JAG69642.1), *Monodelphis domestica* (XP_001371216.2), *Sarcophilus harrisii* (XP_012408864.1). **(C)** The MASP2 phylogenetic tree was constructed using the *Phascogale calura* MASP2 sequence obtained from this study, along with *Homo sapiens* (EAW71674.1), *Mus musculus* (AAH13893.1), *Sus scrofa* (ACR23350.1), *Monodelphis domestica* (XP_016288752.1), *Sarcophilus harrisii* (XP_012401015.1). All sequences were obtained from GenBank.

4.3.5 Quantitative Real-Time PCR (qPCR)

All cDNA synthesised using 1 μg of RNA was diluted 10-fold before qPCR use. The qPCR mix was prepared to give a total volume of 25 µL: 6.5 µL water, 2.5 µL primers (forward and reverse; 10 µM), 1 µL cDNA, and 12.5µL of Rotor-Gene SYBR Green PCR Master Mix. The following amplification program was used: 5 min denaturation at 95°C, 40 cycles of amplification with 5 s at 95°C (denaturation), 10 s at 60-65°C (depending on annealing temperatures listed in Table 4.3), and 15 s at 72°C (elongation). Annealing temperatures were optimised according to individual genes and primers. A melting step was performed to confirm a single gene-specific peak by a stepwise increase in temperature ranging from 60°C to 95°C at a ramp rate of 1°C/s. All samples were run in triplicate.

Amplicon specificity and size were evaluated by running qPCR products in a standard 2% agarose gel electrophoresis and analysing the product as previously described. Standard curves were analysed for each complement gene being measured to obtain amplification efficiencies (*E*) using adult liver cDNA. *E*-values for each gene were assessed using five-fold cDNA dilutions.

The relative expression ratio (*R*) of the target genes were calculated based on *E*-values and the Cq deviations (ΔCq) of the unknown samples versus calibrators, β-Actin and GAPDH. The Pfaffl method was used to analyse and compare the expression levels of different age groups (Pfaffl, 2001) using qBase^{PLUS} software (Hellemans et al., 2007). The Pfaffl method was chosen due to the equation's ability to have varying qPCR amplification efficiency values. The quantification cycle (Cq) was automatically determined for each reaction by the Rotor Gene Software (v. 1.7.94). Data were analysed using the following equation:

$$Ratio = \frac{Efficiency(\text{target})^{\ \Delta Cq \text{ target (calibrator}-\text{sample)}}}{Efficiency(\text{reference})^{\ \Delta Cq \text{ reference (calibrator}-\text{sample)}}}$$

4.3.6 Statistics

The average relative quantities of C3, C1r, CFP and MASP2 expression were calculated for pouch young, juveniles and adults. Data were averaged for different age groups, and expressed as means ± SD. Trends for qPCR were analysed by one-way ANOVA. Pairwise comparisons were made between pouch young, juveniles and adults using Fisher's LSD test ($p < 0.05$ considered significant).

4.4 Results

4.4.1 Sequence Identification of Complement Components

Initial PCRs to amplify partial C3, CFP and MASP2 sequences were successful. The amplicons for C3, CFP and MASP2 were evident based on the strong fluorescence observed, therefore indicating readily detectable gene expression. Altogether, we amplified 3625-bp of C3, 253-bp of CFP and 852-bp of MASP2 and a search in BLASTN (GenBank) using these red-tailed phascogale sequences as the query confirmed their identity as C3 and MASP2 respectively. Red-tailed phascogale C3 sequence obtained an e-value of 0.0 and sequence coverage of 3078/3588 (86%) with tammar wallaby (*Macropus eugenii*) C3 (AAW69835.1), and an e-value of 0.0 and sequence coverage of 2990/3565 (84%) with gray short-tailed opossum C3 (XP_003340773.1). Red-tailed phascogale CFP obtained an e-value of 1e-113 and sequence coverage of 235/253 (93%) with the Tasmanian devil (XM_012553410.1), and e-value of 2e-85 and sequence coverage of 211/251 (84%) with the gray short-tailed opossum

(XM_016426811.1). Red-tailed phascogale MASP2 sequence obtained an e-value of 0.0 and sequence coverage of 741/768 (96%) with Tasmanian devil MASP2 (XM_012545561.1), and an e-value of 0.0 and sequence coverage of 662/808 (82%) with gray short-tailed opossum MASP2 (XM_007491443.1).

A phylogenetic tree indicates the divergence of complement sequences (Figure 4.1). Sequences and lengths of both red-tailed phascogale C3 and MASP2 sequences were compared to several eutherian and marsupial sequences (C3, Supplementary Material 4.1; CFP, Supplementary Material 4.2; MASP2, Supplementary Material 4.3). All three sequences have been submitted into GenBank with the accession numbers KX101055 (C3), KX151185 (CFP) and KX101056 (MASP2).

Only 172-bp were obtained for C1r using the sequence obtained from qPCR (Genbank accession number KX151184). From this result, red-tailed phascogale C1r obtained an e-value of 2e-71 and sequence coverage of 165/172 (96%) with the Tasmanian devil (XM_012550743.1), and e-value of 9e-50 and sequence coverage of 149/172 (87%) with the gray short-tailed opossum (XM_007503684.1).

Five different motifs were found for C3 cDNA; EGF-like domain signature 1 (EGF), C-terminal cystine knot signature (CTCK), 2Fe-2S ferredoxin-type iron-sulfur binding region signature (2Fe-2S), von Willebrand factor type-C domain signature (vWF) and thiolases active sites. Further analysis of red-tailed phascogale C3 cDNA also revealed additional motifs and binding sites. As C3 is a complement protein involved in Classical, Alternative and Lectin Complement pathways, binding sites for CR1-3, complement factor H (CFH), CFB and CFP were detected. In addition, C3a and complement factor I (CFI) were identified in the C3 sequence, emphasizing the multiple functions of the protein (Supplementary material 4.4). The MASP2 sequence had three different motifs including vWF, 2Fe-2S and

thiolases active sites (Supplementary material 4.5), and the CFP sequence had five motifs including EGF, vWF, 2Fe-2S, a mammalian defensin signature and an anaphylatoxin domain signature, along with a GAG-binding site (Supplementary material 4.6). Functional domains, including binding sites, are included in Additional File 4, 5 and 6 for C3, MASP2 and CFP, respectively.

4.4.2 Complement Expression in Red-tailed Phascogales

All expressed sequences were confirmed via cloning and sequencing. Expression of pouch young from ages 1 to 18 days were compared separately to 31-day pouch young, juveniles and adults because of the different tissues used (1-18 days, whole bodies; 31-day pouch young, juveniles and adults, liver). On average, the complement expressions in whole body tissues between 1-day and 6-18 day old pouch young for all complement genes showed significantly higher expressions in older individuals. Average C3 expression increased from 1-day to 6-day individuals and remained relatively constant from ages 6- to 18-days. In contrast to C3, C1r, CFP and MASP2 expression fluctuated in pouch young from ages 6- to 18-days. Comparing the youngest (1-day) and oldest (18-days) whole body samples, C1r, CFP and MASP2 expression significantly increased between the two age groups ($p < 0.05$; Figure 4.2).

According to the results using liver samples, pouch young expression of C3, C1r and MASP2 continued to increase until individuals become juveniles (5-months old). The individual averages of C3, C1r and MASP2 expression in liver tissues of 31-day pouch young were significantly lower than juveniles ($p < 0.05$). In contrast, average CFP expression was higher in pouch young because of an individual 31-day liver sample with the highest CFP

expression in this study. All of the complement proteins in this study decreased in expression levels from juveniles to adults ($p < 0.05$; Figure 4.2).

4.5 DISCUSSION

Marsupial populations are often affected by parasites and diseases (reviewed in Obendorf, 1993), including various mycobacterial infections that have affected the Tasmanian devil (Michael and Sangster, 2010) and rufous hare-wallaby (*Lagorchestes hirsutus*) (Young et al., 2003). While disease susceptibility implies these marsupials may lack a subcomponent of the innate immune system, previous studies have found marsupials to have an effective innate immune system from birth to adulthood (Joss et al., 2009; reviewed in Old and Deane, 2000), which prevents the effects of infectious diseases. Therefore, it is worth identifying different aspects of the innate immune system,

We have previously reported functional data (based on serum studies) suggesting that both the Classical and Alternative pathways are active in red-tailed phascogales (Ong et al., 2015), but due to the lack of marsupial-specific anti-C1q needed to inhibit C1q binding (Roos et al., 2003), we were unable to specifically identify the presence of the Lectin Complement pathway. In the current study, we focussed on MASP2 isolation and expression, as it is an important pathway-specific molecule, whose expression implies an active Lectin Complement pathway. The pathway recognises the carbohydrates on the surface of pathogens and activates the coagulation system (reviewed in Matsushita et al., 2013). The identification of MASP2 itself is important as the protein protects the body against infections (Ali et al., 2012; Fuchs et al., 2011). In addition, MASP2 cleaves prothrombin to form thrombin, creating fibrin clots that allow microbes to be contained (Krarup et al., 2007).

Like MASP2, the complement component, C3, was also chosen for investigation because of its importance in activating the complement system and its central role in the three main complement pathways found in eutherians (Sarma and Ward, 2011). The presence of C3 has been established in other marsupials, including the Northern brown bandicoot (*Isoodon macrourus*; Baker et al., 2007), tammar wallaby, Tasmanian devil and koala (*Phascolarctos cinereus*; Ong et al., 2016a).

The C3 and CFP proteins had both EGF and vWF motifs, that are well-recognised in complement family molecules (reviewed in Ong et al., 2016a). While EGFs have a role in calcium-binding, an important factor for complement as both Classical and Lectin pathways are calcium-dependent (Takahashi et al., 1999; Zhang et al., 1999), they also play an important role in Complement Factor B (CFB) synthesis (Circolo et al., 1990). The vWF motif is able to deactivate the complement system by acting as a cofactor for factor I-mediated cleavage of C3b, a subcomponent of C3 (Feng et al., 2015).

The vWF motif is present in the red-tailed phascogale MASP2 cDNA sequence (Additional File 4.2), however the EGF motif, present in eutherian MASP2 cDNA sequences (Fujita, 2002) was absent. It is however important to note that only a partial sequence of MASP2 was obtained in this study, therefore further studies are required to provide a full sequence of red-tailed phascogale MASP2, which may support the presence of this motif.

In complement expression, C1r, CFP and MASP2 complement proteins were specifically chosen for the expression study because of their explicit involvement with the Classical, Alternative and Lectin Complement pathways, respectively (Sarma and Ward, 2011). The qPCR method is used for measurement of complement gene expression, and liver was the tissue of choice for this study because it is the main site of complement synthesis in

eutherians (Sarma and Ward, 2011). Unfortunately, liver could not be extracted from recently born pouch young due to their small size, therefore whole body tissues were used.

The C1r component is involved in the activation of the Classical Complement pathway (Sarma and Ward, 2011). It is part of the C1 complex, involving the attachments of C1q, C1r and C1s, which binds to the Fc regions of either IgG or IgM (Sarma and Ward, 2011). The activation of C1r eventually leads to the activation of the Classical Complement pathway. The function of MASP2 is similar to C1r, as the MASP2 activation by pathogen binding also leads to the cleaving of C2 and C4 (Fujita, 2002). In humans, the deficiency of both C1r and MASP2 makes them more prone to the development of bacterial infections (Sarma and Ward, 2011; Stengaard-Pedersen et al., 2003). If the presence and expression of MASP2 were confirmed in marsupials, it would indicate that the Lectin Complement pathway possibly aids bacterial protection in pouch young, particularly when they are exposed to bacteria in the pouch, and lack a functional adaptive immune system shortly after birth. Protection from bacteria could also be provided by CFP.

The Alternative pathway specific protein, CFP, plays a role in the positive regulation of the Alternative Complement pathway (Bustin et al., 2009; Sarma and Ward, 2011). Regulation by CFP occurs through its ability to bind to C3- and C5-convertase enzyme complexes, which are constantly activated. By binding to those complexes, it splits C3 and C5 components resulting in the inactivation of the Alternative Complement pathway, subsequently avoiding autoimmune diseases (Bustin et al., 2009).

The complement expression levels of C1r, CFP and MASP2 increased between 1 to 7 day olds, decreased in 10-day olds and increased again in 18-day olds, unlike the expression of C3 (Figure 4.2). The reason for this is unknown, but an increase in complement expression can occur due to undetected injury, inflammation and other diseases (reviewed in Anderson

et al., 2010). However, we believed that all individuals were healthy at the time of euthanasia. Fluctuation patterns of expression could also be attributed to the difficulty in attaining pouch young ages. Pouch young ages in this study were determined using crown-rump length. As the growth rates of pouch young depend on the litter (Serena and Soderquist, 1988; Stannard and Old, 2015), it may have influenced the outcomes of relative expression. We can confirm that 1-day old individuals were the youngest pouch young sampled, and therefore results showing a significant increase in expression from 1-day olds to ages 6-18 day olds suggests the development of the complement system as pouch young mature. The gender could also have influenced complement expression, as it was not possible to macroscopically determine pouch young gender at a young age.

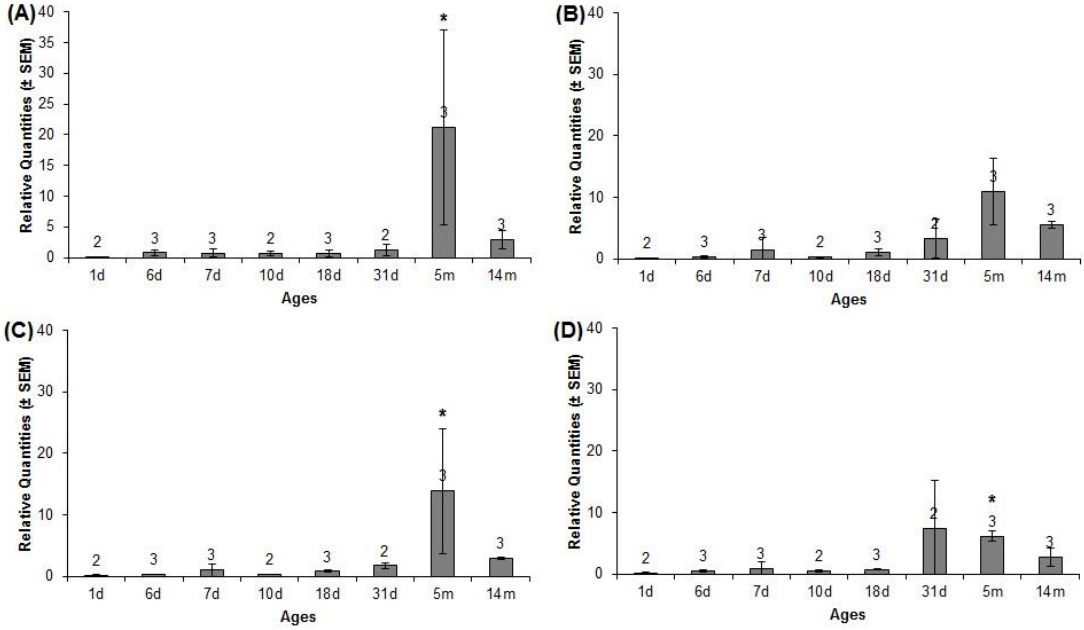

Figure 4.2 Mean expression data for (A) C3, (B) MASP2, (C) C1r and (D) CFP at different age groups normalised against β-Actin and GAPDH. Ages are according to days (1, 6, 7, 10, 18 and 31), except for five month olds (labelled 5m) and 14 month olds (labelled 14m). Sample sizes for each age group are listed above each bar. Error bars indicate standard deviation (SD) of samples run in triplicate. An ANOVA test comparing the statistical differences between five and 14 month red-tailed phascogales are indicated by asterisks (P <0.05). Primer sequences and amplicon sizes are provided in Table 4.2. The crossing-threshold values ranged from ~26 to ~39.

It would have been helpful if we knew the source of complement in marsupial pouch young, whether it was from cells in the milk or a specific organ in the pouch young body. Unfortunately, it was difficult to isolate organs because younger pouch young were small in size. As complement expression was detected in whole body tissues of pouch young ages 1-18 days old; it is possible for complement to originate from a tissue other than liver. It is possible for complement proteins and other complement-related proteins to be passed from mother to pouch young. Very Early Lactational Protein (VELP) in marsupial milk is found to have similar functions to mannose-binding lectin (MBL) protein, and has been identified in brushtail possum (*Trichosurus vulpecula*) milk at days 17-71 lactation (Kuy et al., 2007) and

tammar wallaby milk at 40 days lactation (Joss et al., 2007). Complement B protein was identified in tammar wallaby milk at 72-172 days lactation (Joss et al., 2009).

While suckling, marsupials start to develop their own immune system (Deane and Cooper, 1988), therefore it is possible for pouch young to develop their own complement system before they are fully weaned. The comparison between most pouch young samples with juvenile and adults could not be made because we used pouch young whole bodies as samples, instead of liver. We did manage however to opportunistically obtain two 31-day old individuals to represent pouch young expression in liver tissue. The average pouch young C3, C1r and MASP2 expression increased as they matured into juveniles, indicating an increased use of the complement system as pouch young become older. This result is in contrast with CFP expression, where it decreased in juveniles, because a 31-day old sample had a particularly high CFP expression compared to the other individual of the same age (Figure 4.2). The results could be affected by an undetected injury, causing an increase in CFP expression. As there are only two individuals tested in the 31-day (pouch young) age group, further studies are required to investigate the expression of CFP in developing marsupials.

The juveniles (5-months) showed an average expression higher than adults (14-months), and the results were consistent for all complement components in this study. Results possibly indicate the suppression of immune and inflammatory reactions in post-mating captive male red-tailed phascogales. As male red-tailed phascogales used in this study were held in captivity, we were able to confirm that the sampled males have all been through a mating process during the breeding season and were subsequently unable to reproduce (Kerr and Hedger, 1983; Millis et al., 1999). As animals in this study were opportunistically obtained, we could not obtain enough female samples for an expression study. Therefore, the link

between complement and semelparity has not been investigated specifically and should be considered in future studies.

The results of this study confirm the presence of expressed C3, C1r, CFP and MASP2 in all age groups (Figure 4.2), as evidenced by sequence identity, including specific motifs, and the likely presence of a Lectin Complement pathway. Relative expression of these genes has provided insight into the development of the complement pathway in young marsupials. We encourage further research to explore the source of complement gene expression in very young animals.

4.6 CHAPTER 4 REFERENCES

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4.7 CHAPTER 4 SUPPLEMENTARY MATERIALS

Additional Supporting Information may be found in the appendix:

Supplementary Material 4.1 Primers used for C3, CFP and MASP2 sequence identification using RACE-PCR.

Supplementary Material 4.2 Primers used in qPCR for gene expression of complement components C3, C1r, CFP and MASP2. The two reference genes used for this study were β-Actin and GAPDH.

Supplementary Material 4.3 Efficiency and R^2 data for genes used in this study, and the optimised annealing temperature.

Supplementary Material 4.4 Protein sequence alignments of red-tailed phascogale complement component 3 (C3) with other mammalian C3 protein sequences. Highlighted area shows 80% similarity between C3 sequences. These genes are abbreviated using the first letter from the genus and the first two letters of the species name followed by the gene name. Along with the red-tailed phascogale sequence, the alignment was performed using C3 sequences of: *Homo sapiens* (AAA85332.1), *Mus musculus* (AAH43338.1), *Sus scrofa* (AAG40565.1), *Isoodon macrourus* (ABC84125.1), *Monodelphis domestica* (XP_003340773.1), *Macropus eugenii* (AAW69835.1), *Sarcophilus harrisii* (XP_012397519.1).

Supplementary Material 4.5 Protein sequence alignments of red-tailed phascogale mannan-binding lectin serine protease 2 (MASP2) with other mammalian MASP2 protein sequences. Highlighted area shows 80% similarity between MASP2 sequences. These genes are abbreviated using the first letter from the genus and the first two letters of the species name followed by the gene name. Along with the red-tailed phascogale sequence, the alignment

was performed using MASP2 sequences of: *Homo sapiens* (EAW71674.1), *Mus musculus* (AAH13893.1), *Sus scrofa* (ACR23350.1), *Monodelphis domestica* (XP_016288752.1), *Sarcophilus harrisii* (XP_012401015.1). All sequences were obtained from GenBank.

Supplementary Material 4.6 Protein sequence alignments of red-tailed phascogale complement factor properdin (CFP) with other mammalian CFP protein sequences. Highlighted area shows 80% similarity between CFP sequences. These genes are abbreviated using the first letter from the genus and the first two letters of the species name followed by the gene name. Along with the red-tailed phascogale sequence, the alignment was performed using CFP sequences of: *Homo sapiens* (AAB62886.1), *Mus musculus* (EDL00734.1), *Sus scrofa* (JAG69642.1), *Monodelphis domestica* (XP_001371216.2), *Sarcophilus harrisii* (XP_012408864.1). All sequences were obtained from GenBank.

Supplementary Material 4.7 The nucleotide and deduced amino acid sequence of complement component 3 (C3) in the red-tailed phascogale (*Phascogale calura*). EGFs are bold, 2Fe-2S ferrodoxin motif is underlined, thiolases active sites have wave underlines, thioester sites have dashed underlines, C-terminal cysteine knot signature have dotted underlines. von Willebrand factor (vWF) domain signatures are in grey. Various binding sites have double underlines and are labelled in figure.

Supplementary Material 4.8 The nucleotide and deduced amino acid sequence of mannan-binding lectin serine protease 2 (MASP2) in the red-tailed phascogale (*Phascogale calura*). The 2Fe-2S ferrodoxin motif is underlined, thiolases active sites have wave underlines, and the on Willebrand factor (vWF) domain signature is double underlined.

Supplementary Material 4.9 The nucleotide and deduced amino acid sequence of complement factor properdin (CFP) in the red-tailed phascogale (*Phascogale calura*). EGFs are bold, anaphylatoxin domain signature motif is underlined, Integrin-β is in italics and von

Willebrand factor (vWF) domain signature is in grey. GAG-binding site have double underlines, and the start and end of the defensin sequence are labelled in figure.

CHAPTER FIVE

DETECTION OF AN ACTIVE COMPLEMENT SYSTEM IN RED-TAILED PHASCOGALES (*PHASCOGALE CALURA*)

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5.1 Chapter Outline And Authorship

Chapter 5 investigated the function of the complement system in red-tailed phascogales. The detection of a functional complement system indicated that marsupials utilises the complement system to destroy or eliminate pathogens, similar to eutherians. The study was conducted with the approval of the Western Sydney University's Animal Care and Ethics Committee, A9872.

The following manuscript is jointly authored, where I am the primary author and performed blood collection, serum extractions, laboratory analysis, and drafted the manuscript. Dr. Lauren Young provided analytical and editorial feedback on manuscript drafts, which aided in the development of the manuscript. Associate Professor Julie Old supervised the development of the study, provided editorial feedback on manuscript drafts and acted as the corresponding author of the manuscript.

The authors of this manuscript would like to thank the editor and anonymous reviewers who provided critical feedback on the manuscript prior to being accepted and published by the journal. This chapter is a published journal article and should be cited as:

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5.2 INTRODUCTION

The marsupial immune system is now known to have a high level of complexity (Wong et al. 2006) but there is still a general lack of fundamental information in this research area. Fundamental data such as information on complement proteins and their function, taken for granted in eutherian mammals, has not been collected for many marsupials due largely to a lack of proven clinical and laboratory assays that are appropriate for such assessments. Recently, this has begun to be addressed as the genome sequence data for the gray short-tailed opossum (*Monodelphis domestica*) (Mikkelsen et al. 2007), tammar wallaby (*Macropus eugenii*) (Renfree et al. 2011) and Tasmanian devil (*Sarcophilus harrisii*) (Murchison et al. 2012) became available and we have identified some of the major complement proteins at the gene level in these three marsupial species (Ong et al. 2016).

The complement system is one of the major components of humoral immunity; providing one of the first defences against pathogens once epithelial barriers are breached. Comprised of a family of proteins that circulate in serum in inactive forms, complement proteins are proteolytically cleaved and activated in the presence of pathogens (alternate pathway), by antigen-antibody complexes (classical pathway) or by lectins associated with carbohydrates on the invading microbe's surface (lectin-binding pathway) (Janeway et al. 2001).

As eutherian mammals have an established complement system (Sarma and Ward 2011), we would expect that metatherian mammals (the marsupials) also possess a complement system as part of their immune portfolio. Previously, haemolytic assays performed on the serum of two marsupials, the Virginian opossum (*Didelphis virginiana*; (Wirtz and Westfall 1967) and the gray short-tailed opossum (Croix et al. 1989; Koppenheffer et al. 1998) suggest that there is a functional complement system in members

of the opossum family, but there have been no other studies investigating complement activation in other species of marsupials using this method; a method that is routine for the clinical assessment of complement in humans and other eutherian mammals (Bradley 1997). Investigations of the presence and function of a complement system in marsupials is important as it could be the key to understanding the mechanism of immune protection of pouch young (Joss et al. 2007). For example, we know that immunoglobulins (antibodies) form part of passively acquired immunity in neonatal marsupials (Joss et al. 2007), so it is reasonable to assume that if complement proteins play a role in immune protection of the young, then the complement pathway associated with antibody-antigen complexes will be present and functional. Therefore, we sought to determine whether the classical complement pathway is utilised in the model marsupial, the red-tailed phascogale (RTP; *Phascogale calura*).

RTPs are small nocturnal dasyurid marsupials (Family Dasyuridae) (Bradley 1997) with populations restricted to the south-west of Western Australia (Berden et al. 1978; Kitchener 1981). The complexity of the marsupial immune system, along with the population decline of most dasyurid species since European settlement, enhances the importance of the RTP as a model species for this study. As of 2003, at least 24% of small dasyurid species (<500 g) were declared vulnerable, endangered or data deficient (Wilson et al. 2003), so investigation of a dasyurid complement system would enhance our understanding of the immune system of these and other dasyurid marsupials.

Evidence for a complement system in eutherian mammals can be determined using haemolytic complement assays. These assays are conducted using serum as a source of complement together with unsensitised rabbit erythrocyte (RbE) and sensitised sheep erythrocytes (SE) as indicator cells (Bradley 1997). The lysis of unsensitised RbE and

sensitised SE when exposed to dasyurid serum will confirm the presence of a classical and alternative complement pathway, respectively, in this species and enhance our capacity to investigate fundamental immunity in dasyurid mammals.

5.3 Materials And Methods

5.3.1 Animals

Male and non-pregnant female RTPs between ages four months to one year old were opportunistically captured and euthanased at the University of Western Sydney, Hawkesbury campus during routine colony maintenance (UWS Animal Care and Ethics approval A9872). The small size of the animals (<80g) and the limited volume of blood available for analysis required the collection of whole blood via cardiac puncture immediately after euthanasia. Blood was collected from RTPs via heart puncture using a 2 mL syringe and 23-gauge needle. Blood was quickly transferred into a sterile 1.5mL Eppendorf tube, and allowed to clot for one hour at room temperature. Blood clots were removed by centrifugation at 500 RCF for 10 min. The serum collected was stored in the -80°C freezer until use. Male and female RTP sera were pooled separately before use.

RbE was collected via the marginal vein of the ear of a New Zealand white rabbit (*Oryctolagus cuniculus*) and immediately placed into 50% Alsever's solution (v/v). The rabbit was restrained in a towel before the phlebotomy procedure, and the ear was anesthetised locally with a lidocaine containing cream, EMLA®, as per UWS Animal Care and Ethics approval A10534. After collection, RbE was stored at 4°C. Before each assay, RbE were washed twice using Alternative Pathway buffer (AP buffer; gelatin veronal buffer containing 5 mM Mg^{2+} and 5 mM ethylene glycol-bis[β-aminoethyl ether]N,N'-tetraacetic acid (EGTA)) and resuspended in AP buffer to make a final concentration of 1% (v/v).

5.3.2 Alternative Complement Pathway Assay

To test the presence of an alternative complement pathway, the serum of male and female RTP serum were pooled and standard haemolysis tested as per Morgan (2000). The assay was conducted using 96-well round (U) bottom assay plates (Corning Costar #3799, NY). Serial dilutions of serum in AP buffer with concentrations of 1:10, 1:5, 1:4, 1:3, 1:2, 1:1, 2:1, 3:1, 4:1, 5:1, 10:1 and 10:0 was set up with a total of 50 µL/well.

After RTP serum was added to each well, 50 µL of 1% RbE (v/v) was added to the wells, halving serum dilutions. The plate was sealed with ParafilmM Laboratory Film (Pechiney Plastic Packaging, Neenah, WI) and covered with a lid. The plates were gently shaken for 10 s and incubated at 37°C for 30 min with intermittent shaking. After incubation, 50 µL of ice-cold N-saline (9 g NaCl in 1 L H_2O) was added to the wells to stop the reaction. The plates were centrifuged at 5000 RCF for 5 min and 100 µL of supernatant was extracted and placed into a flat (F) bottom cell culture plate (Cellstar® #655980). The reactions were measured using a Benchmark Plus Microplate Reader (BioRAD Laboratories) at 412 nm.

Samples are incubated for 30 min except for the kinetic assay, which ran for 20, 40, 60 and 80 min with 0%, 10%, 25% and 50% (v/v) serum concentration. The effects of different temperatures were also measured using 50% (v/v) serum concentrations for both male and female RTP. All samples were run in triplicates for reliable results.

5.3.3 Classical Complement Pathway Assay

Whole sheep blood was obtained from Serum Australis (Manilla, New South Wales, Australia). SE were prepared by washing three times in veronal buffered saline with 0.1% gelatin (GVBS) and resuspended in veronal buffered saline containing gelatin and EDTA (GVBS-EDTA). Resuspended SE were sensitised using rabbit anti-sheep red blood cell

stroma (Sigma-Aldrich, Milwaukee, WI, USA) and were resuspended again to a final concentration of 2% (v/v) sensitised SE.

An initial optimisation to estimate the serum concentration required for 50% SE lysis was conducted. Serum from a male animal was chosen to optimise the assay to avoid possible biological variation in female serum due to reproductive status. Serum (50 μL) from the one male RTP was incubated at 34.7°C (Pusey et al. 2013), and a further 50 μL serum was incubated at 56°C for one hour to inactivate serum complement and act as a negative control. After incubation, the haemolytic assay was performed in 96-well (eight rows by 12 columns) round (U) bottom assay plates by transferring 25 μL of serum into columns 1 and 2 of the plate and 25 μL of veronal-buffered saline containing 0.1% gelatin (GVBS) to columns 2 to 12. The contents from column 2 were then serially diluted through to column 12 resulting in dilutions ranging from 1 (Column 1) to 1/2048 (Column 12).

After the serum was added to each well, 25 μL of 2% (v/v) sensitised SE was added to all wells, halving all serum dilutions. The plate was sealed with ParafilmM Laboratory Film and covered with a lid. Plates were gently shaken for 10 s and further incubated at 37°C for 90 min with intermittent shaking. After incubation, 100 μL of cold GVBS was added to all wells to stop the reaction. The plates were centrifuged at 5000 RCF for 5 min. Subsequently, 100 μL of the supernatant was extracted and placed into a flat (F) bottom cell culture plate. The new plate was visually assessed for lysis before spectrophotometric measurement at 540 nm in a Benchmark Plus Microplate Reader. The plate was also measured at 412 nm to allow data from this study to be compared to an earlier study on marsupial haemolytic assays (Koppenheffer et al. 1998).

After initial optimisation using the results obtained from male RTP serum, the range of serum dilutions that obtained a 50% SE lysis was determined for the haemolytic assay.

Samples from four other RTPs (three male and one female) were then analysed using these conditions. The number of replicates for each animal ranged from three to five, depending on the volume of serum available (Male 1, four replicates; Male 2, three replicates; Male 3, four replicates; Female, five replicates).

5.3.4 CONTROLS, STATISTICS AND CALCULATIONS

Controls include a no complement reaction (50 µL AP buffer; 50 µL RbE/SE) and a 100% lysis reaction (50 µL H_2O; 50 µL RbE/SE), which were included in each plate for every assay reaction, with 50 µL of N-saline added at the end of its incubation. The percentage of RbE/SE haemolysis of all samples were calculated as:

$$\text{Haemolysis (\%)} = \frac{OD_{(x)}\text{ haemolytic test} - OD_{(x)}\text{ no complement reaction}}{OD_{(x)}\text{ 100\% lysis} - OD_{(x)}\text{ no complement reaction}} \times 100$$

where OD is optical density and x is 412 nm and 540 nm for alternative and classical complement assay, respectively. Results from the equation were averaged and graphed with standard deviations.

For the classical complement assay, the average measurements for replicates within each serum dilution were calculated for each individual animal. One-way analysis of variance (ANOVA) was applied to determine the significant differences between replicates at each serum dilution, to ensure that resulted between individuals were statistically similar.

5.4 RESULTS

5.4.1 ALTERNATIVE COMPLEMENT PATHWAY ASSAY

Figure 5.1 shows the successful haemolysis of RbE by components of RTP serum. For both male and female RTPs, higher concentrations of serum effectively lysed a higher percentage of RbE. The response of RbE to serum can be seen in Figure 5.1 and 5.2, where reactions with higher serum concentrations exhibited higher RbE haemolysis percentage.

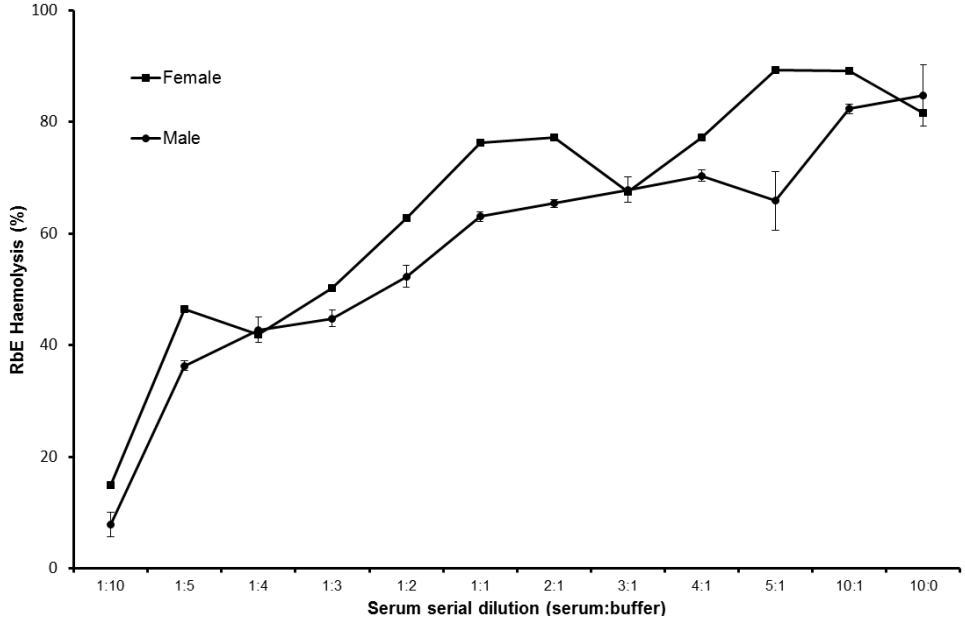

Figure 5.1 Serial dilutions for pooled red-tailed phascogale serum (mean±standard deviation) of males and females (ages ranging from 4months to 1 year), exposed to rabbit erythrocyte for 30 min at 37°C. The samples were centrifuged at 5000 RCF for 5 min, and supernatant was measured at the optical density of 412 nm.

There was no major significant effect when incubation times were varied among samples; however, there was an increase in RbE lysis with higher concentrations of RTP serum (Fig. 5.2). The effect of incubation temperatures show that the lysis of RbE was optimal at 40°C in male RTPs and complete by 30°C in female RTP (Fig. 5.3).

5.4.2 Classical Complement Pathway Assay

Similar to other mammals, we found that for each individual animal, RTP serum effectively lysed an optimised level of sensitised SE and that a range of serum dilutions confirmed complement activation in a number of different RTP blood samples. The initial optimisation assay performed using the serum of a one-year old male RTP successfully lysed 50% of the sensitised SE between 1/4 and 1/16 serum dilutions (Fig. 5.4). As a 50% SE lysis is required to show the presence of classical complement pathway activation, this initial optimisation assay confirmed complement activity in the RTP. Inactivated serum complement showed little to no SE lysis (Fig. 5.5).

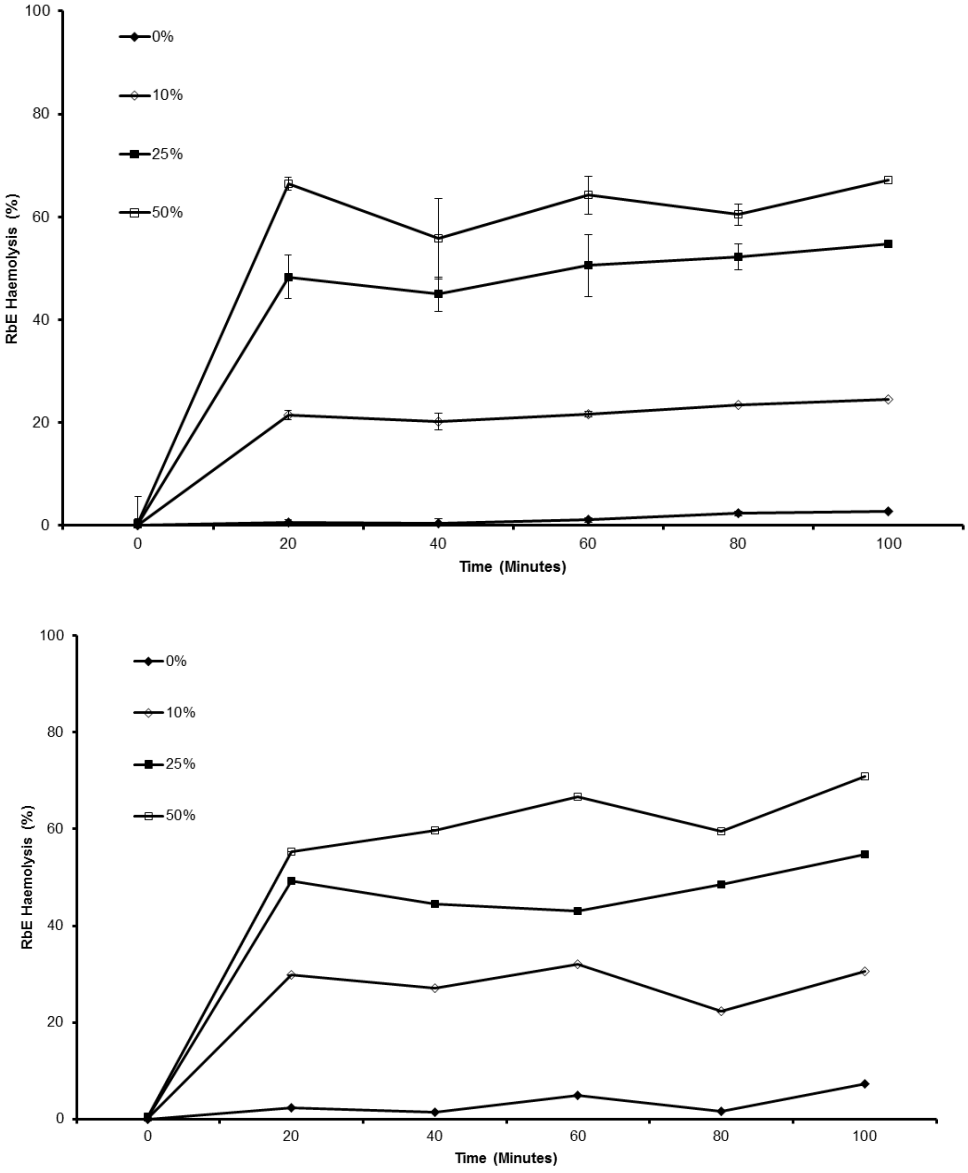

Figure 5.2 Different concentrations of pooled red-tailed phascogale serum of **(A)** males and **(B)** females, exposed to rabbit erythrocyte for varying incubation times. The samples were centrifuged for 5000 RCF for 5 min, and supernatant was measured at the optical density of 412 nm.

As the initial optimisation assay showed 50% SE lysis between serum dilutions of 1/4 and 1/16, subsequent assays were then conducted using serum dilutions in that range. These assays all showed 50% lysis within this serum dilution range, confirming the presence of complement in all animals sampled for this study. To provide comparisons with previously reported data, spectrophotometric measurements at 412 nm in addition to 540 nm were compared and showed similar results, with similar lysis dilution profiles and little to no SE lysis for inactivated serum complement (Fig. 5.5 and 5.6). One-way ANOVAs indicated that each replicate from each serum dilution was not significantly different for the individual animals ($p < 0.05$), however, results between individual animals were significantly different ($p > 0.05$), justifying the use of this assay for individual animal data.

5.5 DISCUSSION

We document the successful application of standard eutherian haemolytic complement assays for functional assessment of dasyurid marsupial serum. Although limited in number, there are reports of the successful use of this assay to investigate complement activity in two species of opossum (Croix et al. 1989; Koppenheffer et al. 1998; Wirtz and Westfall 1967), but our study is the first to report the use of this assay for dasyurid marsupials. Using two well-established functional assays based on complement-mediated lysis of RBC (Matson et al. 2005; Morgan 2000), initial standard testing of haemolytic activity in RTP serum in male and female RTPs indicates the presence of a functional alternative complement pathway (Fig. 5.1) and classical complement pathway in RTPs (Fig.5.3, 5.4 and 5.5). This response is similar to marsupials (Koppenheffer et al. 1998; Wirtz and Westfall 1967) and eutherian

mammals (Klerx et al. 1983), where there is an increase in SE and RbE lysis as serum concentration increases.

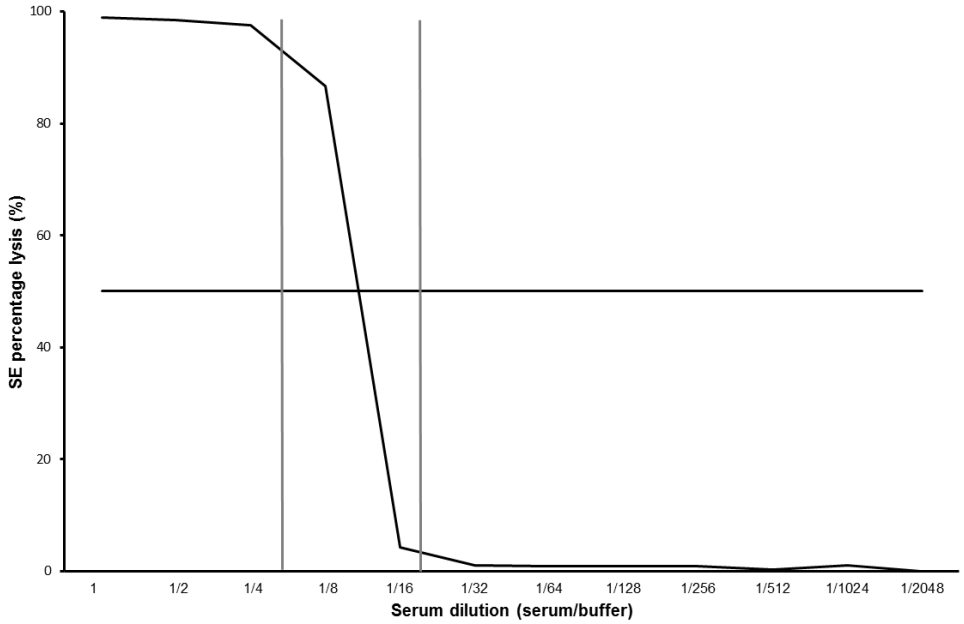

Figure 5.3 Serial dilutions for a one-year old male RTP serum of exposed to sheep erythrocyte for 30 min at 37°C. The samples were centrifuged at 5000 RCF for 5 min and supernatant was measured at the optical density of 540 nm. Serum dilution that successfully lyse 50% (v/v) SE is represented values above the horizontal black line.

The kinetics of RbE lysis for RTP serum showed that RbE lysis varied between samples with different serum concentration (Fig. 5.2). Our results are similar to humans, where RbE haemolysis percentage plateaued before 40 minutes of incubation (Servais et al. 1991). However, the results obtained from the kinetics assay differed from the study by Koppenheffer et al. (1998) on gray short-tailed opossums, which showed that RbE lysis increased up to 60 min of incubation and then plateaued.

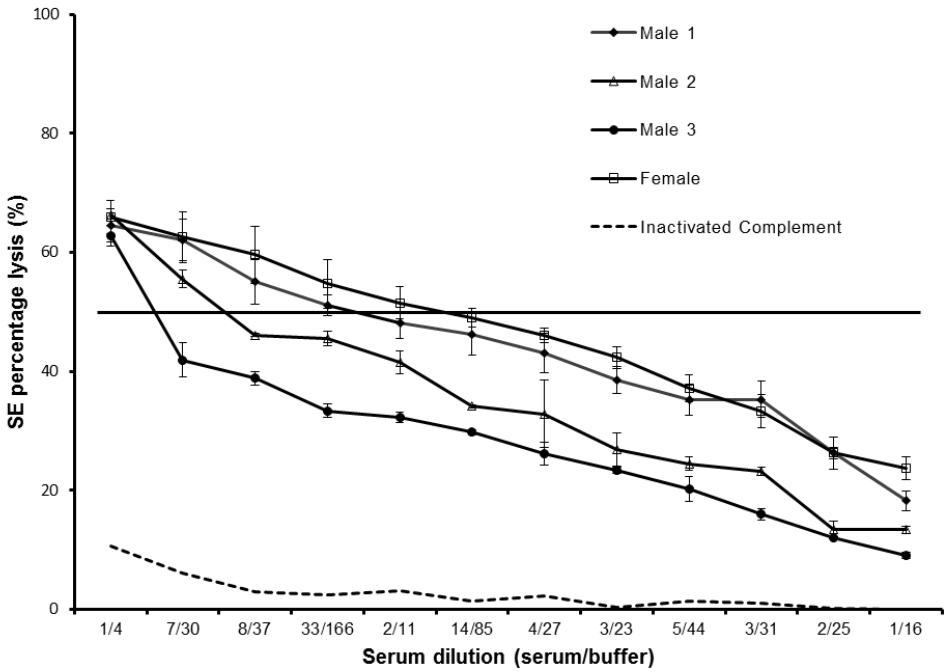

Figure 5.4 The average haemolysis percentage (including standard deviation) for the three male and one female RTP is shown. Male 1 had four replicates, Male 2 and 3 had three replicates and the female had five replicates. Inactivated serum values are included as a representative negative control. The samples were centrifuged for 5000 RCF for 5 min and supernatant was measured at the optical density of 540 nm. Serum dilution that successfully lyse 50% (v/v) SE is represented values above the horizontal black line.

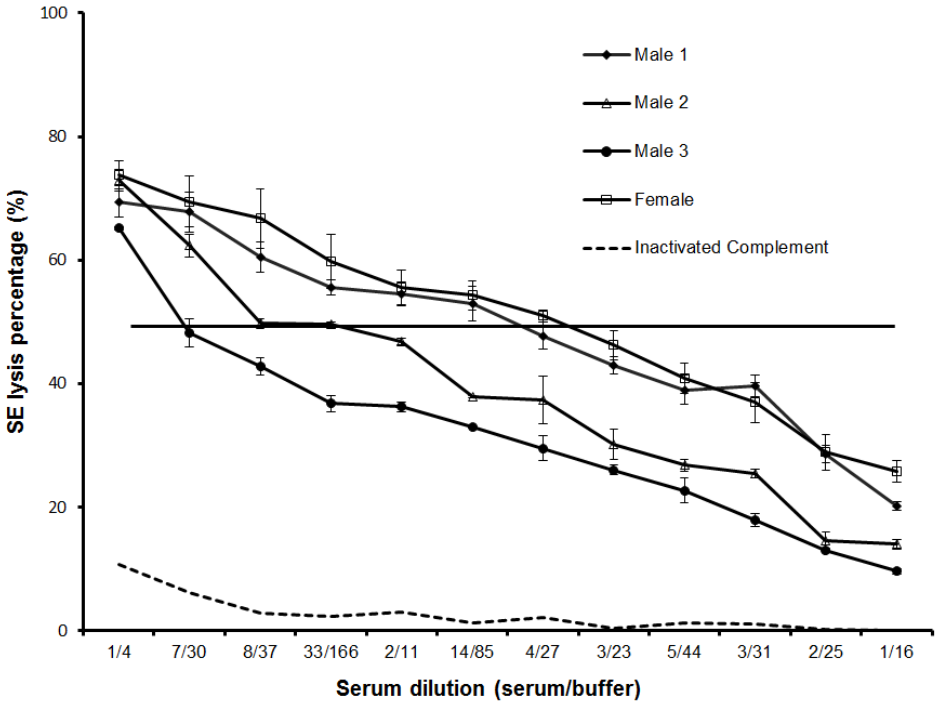

Figure 5.5 The average haemolysis percentage (including standard deviation) for the three male and one female RTP is shown. Male 1 had four replicates, Male 2 and 3 had three replicates and the female had five replicates. Inactivated serum values are included as a representative negative control. The samples were centrifuged for 5000 RCF for 5 min and supernatant was measured at the optical density of 412 nm. Serum dilution that successfully lyse 50% (v/v) SE is represented values above the horizontal black line.

Despite disimilar results when it comes to incubation times, the effect of incubation temperature on RTP serum is comparable to gray short-tailed opossum serum (Koppenheffer et al. 1998). Similar to our study in male RTP serum, the optimal serum dilution for the gray short-tailed opossum showed a continual increase in RbE lysis as temperatures increased (Fig. 5.6). However, there was a slight decrease in RbE haemolysis from temperatures 30°C to 40°C when reacted with female RTP serum, indicating that RbE lysis was complete at approximately 30°C for female RTPs. The optimal incubation temperature between 30°C to 40°C was expected in RTPs as these animals have a body temperature of 34.7°C (Pusey et al. 2013), and a functional alternative complement pathway is expected to function best in the animals' regular physiological body temperature.

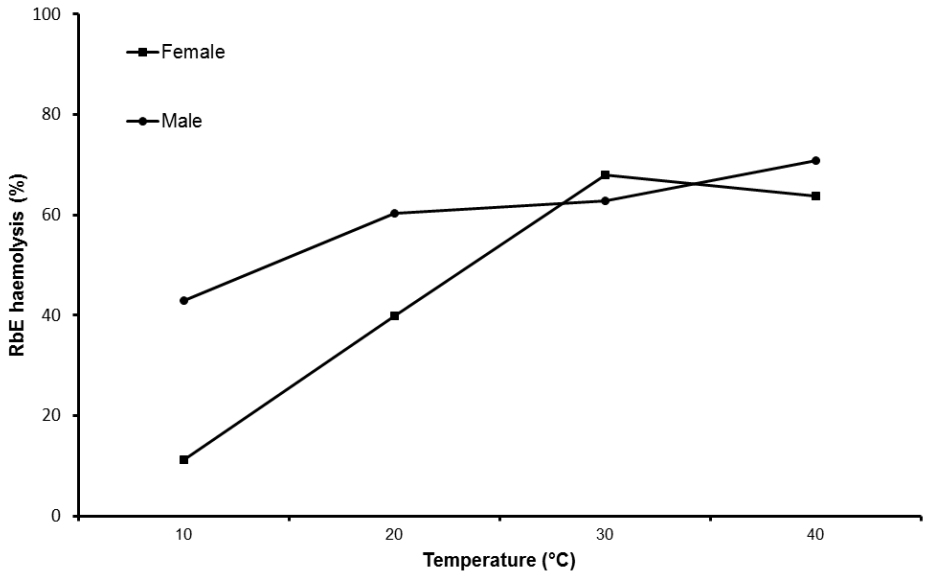

Figure 5.6 Pooled red-tailed phascogale serum dilution of 50% serum (v/v)(mean ± standard deviation) of males and females, exposed to various incubation temperatures ranging from 10°C to 40°C for 30 min. The samples were centrifuged for 5000 RCF for 5 min and supernatant was measured at the optical density of 412 nm.

Similar to the findings in our study, pooled gray short-tailed opossum serum samples at optimal dilutions showed lysis of 50% sensitised SE at temperatures between 25ºC to 40ºC when absorbance was measured at 412nm. However, a 1/500 dilution of gray short-tailed opossum serum demonstrated a SE percentage lysis above 50% (v/v) at 30°C, whereas a serum dilution of at least 2/11 (v/v) (Fig. 5.5) is required for RTP serum to effectively lyse SE by >50%. Whether or not this difference in activity is founded in physiological or experimental differences, both studies confirmed successful inactivation of this lytic activity when the serum was heated at 56ºC; a property of serum complement from humans and other mammals.

Different to the opossum studies, we analysed serum from individual animals for the classical complement assay, rather than pooled serum from a group of animals, which now

allows for comparison of complement activity between different individuals within a species, in addition to comparisons between metatherian and other vertebrate species. As our study involves analysing each individual, our results indicate that 50% sensitised SE lysis occurred at 37ºC between serum dilutions of 1/4 and 1/16, however, the point at which 50% lysis occurred differed between individual animals. This biological variation was expected, and is likely due to differences in individual physiological factors, which we will investigate in future assays from a larger number of animals. Although the female RTP had the highest complement activity, the differences between complement activity in male and female RTPs is currently unknown. As this study was performed using animals that were gained opportunistically during routine colony maintenance, there were no opportunities to obtain further female RTP samples and thus investigate more subtle differences between individuals (e.g. reproductive state, age, and season). Our data will be used to inform such studies in the future.

The results of our study are consistent with a role for the complement system as an immune mechanism that is available to dasyurid marsupials. The presence of functional alternative and classical complement pathways in RTPs show that complement pathways in small dasyurid species are comparable to eutherians such as mice (Bradley et al. 2008), crocodilians such as American alligators (*Alligator mississippiensis*) (Merchant et al. 2007) and other marsupials (Koppenheffer et al. 1998; Wirtz and Westfall 1967), which provides the foundation to further investigate the specific role of complement in the protection of marsupial young.

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CHAPTER SIX

ANTIMICROBIAL ACTIVITY OF RED-TAILED PHASCOGALE (*PHASCOGALE CALURA*) SERUM

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Comparative Immunology, Microbiology and Infectious Diseases; 2017, vol. 51, pp. 41-48

6.1 Chapter Outline and Authorship

Following on from the identification of a functional complement system in previous chapters, Chapter 6 reports the investigation of the antimicrobial properties in red-tailed phascogale serum. Various components in serum are bacterial suppressing agents, with one of them reported to be the complement system. In this study, we tested the effectiveness of serum against four different types of bacteria. The study was conducted with the approval of the Western Sydney University's Animal Care and Ethics Committee, A9872.

The following manuscript is jointly authored, where I am the primary author and performed blood collection, serum extractions, most laboratory analysis, and drafted the manuscript. Jai Green-Barber conducted the Serum Amyloid A (SAA) assays and Anusha Kanuri conducted the haptoglobin (Hp) assays using red-tailed phascogale serum. Anusha Kanuri also isolated Hp and C-Reactive Protein (CRP) in developing red-tailed phascogales. Associate Professor Julie Old and Dr. Lauren Young conceived the initial proposal, provided analytical and editorial feedback on manuscript drafts, which aided in the development of the manuscript. Associate Professor Julie Old supervised the development of the study and acted as the corresponding author of the manuscript.

The authors of this manuscript would like to thank the editor and anonymous reviewers who provided critical feedback on the manuscript prior to being accepted and published by the journal. This chapter is a published journal article and should be cited as:

Ong, OTW, Green-Barber, JM, Kanuri, A, Young, LJ & Old, JM 2017 'Antimicrobial activity of red-tailed phascogale (*Phascogale calura*) serum', *Comparative Immunology, Microbiology and Infectious Diseases*, vol. 51, pp. 41-48.

6.2 INTRODUCTION

The innate immune system defends the body against pathogens shortly after infection has occurred. After detection, specific cells will mediate the removal or destruction of bacteria (reviewed in [1]). Maternal-derived strategies and innate immunity are likely to be particularly important during marsupial development, as these animals are born with no mature immune tissues and unable to mount their own specific immune responses [2, 3]. Studies of immunoglobulin (Ig) isolated from neonatal and foetal serum of tammar wallabies (*Macropus eugenii*) prior to suckling [4, 5], have shown their productionappears to occur immediately or prior to birth, and provides immune protection. The presence of Igs relates to the complement system as Igs identify antigens on bacterial surfaces and subsequently, in eutherian mammals, activates the Classical complement pathway [6, 7].

Mainly synthesised in the liver, complement proteins provide the serum with its antimicrobial properties. Studying marsupial and eutherian mammal immunity, we find that although different, components of the the two immune systems are comparable. Research on marsupial immunity will provide insights into the phylogeny of the immune system, contribute to novel therapies in medical science and aid us in developing an understanding of marsupial disease, and potentially benefit conservation of vulnerable and endangered marsupials.

Studies of marsupial complement are scarce [15-17], however it is important to characterise the complement system in marsupials to identify if it is utilised similarly to their eutherian counterparts. All three marsupial species studied to date have been reported to use the Classical complement pathway, a complement pathway activated using Igs [8]. The Alternative complement pathway has also been detected in the gray short-tailed opossum (*Monodelphis domestica*) [9] and red-tailed phascogale (*Phascogale calura*) [10], showing that the marsupial complement system is able to be activated without the presence of Igs [11].

The Lectin complement pathway, a complement pathway activated by various types of lectins (reviewed in [12]), is also likely present in marsupials based on bioinformatic and gene expression studies [13]. Although complement in red-tailed phascogale serum is able to lyse foreign cells [10], the antimicrobial properties of serum have yet to be reported. Other proteins present in serum, for example lactoferrin and transferrin, also aid in the destruction of foreign cells by destroying bacteria[14].

Acute phase proteins (APPs), such as haptoglobin (Hp), C-reactive protein (CRP) and serum amyloid A (SAA) are produced by the liver [15]. Hps are haemoglobin-binding proteins and iron sequesters that inhibit the growth of microbes by making iron inaccessible [16]. Hp is also important for the synthesis of prostaglandins, leukocyte conscription and migration, and aids in the production of cytokines in response to injury and infection [17]. Hp has been identified in tammar wallaby milk [18], Virginian opossum (*Didelphis virginiana*) [19] and gray short-tailed opossum serum [20] but not in the serum of any dasyurid marsupial.

CRP is also important in the host's defence against infection [21]. CRP is able to activate the Classical complement pathway in eutherians and is involved in phagocytosis [22]. The *CRP* gene has been identified in the gray short-tailed opossum, Tasmanian devil (*Sarcophilus harrisii*) and koala (*Phascolarctos cinereus*) genomes [23] but has not been characterised at either the gene or protein expression levels .

The gene for SAA has only been identified in one marsupial genome: the tammar wallaby [24]. SAA has been reported to activate an inflammasome cascade, promoting the maturation of certain inflammatory cytokines [25]. The level of SAA protein superfamily increases as high as 1000-fold during inflammation, indicating its importance in innate immunity [26].

While antimicrobial activities have been shown to be present in marsupials, the antimicrobial properties of marsupial serum have not been examined previously. The aim of this study was

to focus on the identification of molecules associated with the ability of complement proteins to destroy bacterial cells as part of the innate immune system. Specifically, we sought to identify the gene expression of two APPs (Hp and CRP) and quantify the presence of Hp and SAA proteins in red-tailed phascogale sera using commercially available assays. The red-tailed phascogale was chosen for this study because of its conservation significance, and additional studies on this species would contribute to our understanding of the immunology of vulnerable or endangered dasyurid marsupials. The finding of acute phase proteins (APPs) in the red-tailed phascogale would not only indicate the presence of innate immunity in the species, but also provide evidence that this component of the marsupial immune system is comparable to eutherians.

6.3 Materials and Methods

6.3.1 Sequence Identification of Haptoglobin, C-Reactive Protein and Serum Amyloid A in Marsupials

The presence of human Hp, CRP and SAA were identified by searching the human genome database on GenBank. Similarity searches were conducted using Basic Local Alignment Search Tools (BLAST) [27], nucleotide BLAST (BLASTN) and protein BLAST (BLASTP) in the gray short-tailed opossum, tammar wallaby, Tasmanian devil and koala to identify *Hp*, *CRP* and *SAA* gene homologues in marsupial genomes. For koala sequences, BLASTN and BLASTP were conducted on the Koala Genome Consortium [28] (https://www.koalagenome.org/). Predicted or translated protein sequences were aligned using ClustalW [29] to identify regions of amino acid conservation in marsupials. Sequences from two eutherian species, human and mouse, were used as reference sequences in alignments. A phylogenetic tree was constructed using eutherian and marsupial predicted

protein sequences using Geneious R6.1 by Biomatters (http://www.geneious.com) with the Blosum62 alignment score matrix, a gap open penalty score of 12 and gap extension penalty score of 3, and using the neighbour-joining tree build method. The sequences were simulated on the tree according to the Jukes-Cantor model.

6.3.2 Animals and Sample Collection

Red-tailed phascogales are small, dasyurid marsupials distributed in Western Australia [30]. In this study, we utilised captive-bred red-tailed phascogales housed in the Small Native Mammal Teaching and Research Facility at Western Sydney University, Richmond, NSW, Australia. For further details regarding housing and husbandry refer to [31].

Phascogales were captured and euthanased as per standard protocols approved by the Western Sydney University Animal Ethics Committee (A10534 and A9694). Whole blood was collected in sterile 1.5mL tubes via heart puncture using a 2mL syringe and 23-gauge needle immediately after euthanasia, and centrifuged for 10min at 5,000 rpm (at 4°C). Serum was collected after centrifugation, and kept in the -80°C freezer until required, for a maximum of three months. Altogether, one-year old male (n=5), one year old female (n=5), five month old male (n=6) and three adult female (aged 2.25, 3.25 and 5.25 years) red-tailed phascogales were used for serum collection to determine antibacterial activity of serum, and levels of Hp and SAA (WSU Animal Care and Ethics Approval A9872). To determine antibacterial activity of serum, negative controls heated at 56°C for 30 min were used to inactivate anti-microbial/anti-bacterial proteins. Prior to the experiment, sera were placed in a 37°C incubator.

Tissue samples were opportunistically obtained from animals euthanased for population control reasons (excess breeding stock and older males and females). For the identification of *Hp* and *CRP*, tongue, lung, heart, liver, gastrointestinal tract, spleen and kidney tissues were collected from two adult (male, two years old; female, four years old) and two juvenile (male, 6 months old; female 5 months old) red-tailed phascogales. Liver was collected from pouch young of unknown sex, ages 12, 17, 24, 32 days old, and a 3.5 month old male and 60 day old female. Whole bodies were sampled for pouch young of unknown sex (ages three and seven days old) as they were too small to dissect (Supplementary Material 1). Tissues and pouch young bodies were placed in RNAlater® (Ambion, Austin, TX, USA) and kept in the -80°C freezer until use.

6.3.3 RNA Extraction and cDNA Synthesis

Total RNA of pouch young whole bodies and tongue, lung, heart, liver, gastrointestinal tract, spleen and kidney tissues, were extracted using the SV Total RNA Isolation System (Promega, Wisconsin, USA) as per manufacturer's instructions. cDNA synthesis was performed using the Superscript III First Strand Synthesis System (Invitrogen, California, USA) as per manufacturer's instructions. Final cDNA products were stored in the -20°C freezer until use.

6.3.4 Primer Design and Polymerase Chain Reaction (PCR)

Hp and CRP primers were designed based on predicted cDNA sequences available from GenBank, Tasmanian devil *Hp* (XM_003758541.2) and Tasmanian devil *CRP* (XM_003767868.1), respectively [32]. Primer sequences for *Hp* are as follows: forward primer 5'-GCGGACTGCTGTCTGTAG-3'- and reverse primer 5'-

GGTCTCTTGGTTTCCCAC-3'; and primer sequences for *CRP* are as follows: forward primer 5' CCCAAGACAGTCTCAGGA-3' and reverse primer 5'-CTGCTGTCCCTTTCATGA-3'.

The PCR mix of 20 µL was prepared to give end volumes of: 0.5 µL cDNA, 4 µL x10 GoTaq flexi buffer, 3 µL $MgCl_2$ (3.75 mM), 0.4 µL dNTP (0.2 mM), 1 µL forward primer (5 µM), 1 µL reverse primer (5 µM), 9.8 µL water and 0.3 µL of GoTaq DNA Polymerase (0.075 u/µL) (Promega Corporation, WI, USA). The PCR conditions used were 94°C for 7 min followed by 35 cycles of amplification also at 94°C for 2 min, 50°C for 1 min with a final extension at 72°C for 5 min.

6.3.5 Sequence Analysis

PCR products were evaluated by electrophoresis using 2% agarose gel in TBE. The bands were excised from the gel, ligated into a pCR™2.1-TOPO® vector using the TOPO TA cloning kit (both from Invitrogen, California, USA). Plasmids were cleaned up using Purelink® Quick Plasmid Miniprep Kit (Invitrogen, California, USA) and sequenced at the Australian Genome Research Facility (AGRF), Westmead, Sydney. To assign identity, a similarity search was performed using the Nucleotide Basic Local Alignment Search Tool (BLASTN; Altschul et al. 1990) available on Genbank [32] (http://www.ncbi.nlm.nih.gov/genbank).

6.3.6 Haptoglobin and Serum Amyloid A Assay

Serum for the Hp assay was obtained from a one year old male and a one year old female red-tailed phascogale. The concentration of Hp in serum was determined using the "PHASE"™

Haptoglobin Assay (Tridelta Development Limited, Bray, Ireland) and was conducted as per manufacturer's instruction. As a reference, commercially available rabbit (*Oryctolagus cuniculus*) (Sigma-Aldrich, New South Wales, Australia) and sheep (*Ovis aries*) (Serum Australis, New South Wales, Australia) serum. Samples were analysed using the Benchmark Plus™ microplate reader at 630nm wavelength, and interpreted based on a standard curve of known Hp samples of 0, 0.312, 0.625, 1.25 and 2.5 mg/ml provided by the kit. All standards and samples were performed in triplicate.

Serum for the SAA assay was obtained from six juvenile males (5 months old) and three adult female red-tailed phascogales aged 2.25, 3.25 and 5.25 years old. The concentration of SAA was determined using the SAA kit (LZ Test 'Eiken' SAA- Eiken Chemical Co., Ltd., Tokyo, Japan) and was conducted as per manufacturer's instruction. As a reference, commercially available horse (*Equus caballus*) serum (Vector Laboratories, CA, USA) was also used for a comparison of eutherian and marsupial serum samples. Samples were analysed using the Benchmark Plus™ microplate reader at 570nm wavelength, with a standard curve for each reaction to determine the SAA concentrations.

6.3.7 Bacterial Cultures and CFU Counts

The four bacterial cultures used for the experiment were: *Escherichia coli* (K12), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923) and *Klebsiella pneumoniae* (ATCC 13883). These bacteria, and specifically these strains of bacteria, were chosen as they are commonly used to assess antibacterial activity and are opportunistic pathogens of humans and potentially marsupials. Bacterial cultures were maintained on nutrient agar slants and kept in the 4°C fridge until use. The cultures were inoculated into 10mL sterile Luria broth (LB) and incubated at 37°C overnight in a shaking

incubator at 200 rpm. To ensure that the bacteria were growing at log-phase, the absorbance of the inoculated nutrient broth was measured using the Benchmark Plus™ microplate spectrophotometer (Biorad, Hercules, CA, USA) at 600 OD. A measurement between 0.2 and 1 indicates a log-phase culture.

The bacteria were initially diluted to 1x10^5, and then further diluted by 25% and 50% using sterile LB. The introduction of bacteria and red-tailed phascogale serum was performed in sterile tubes, with a 1:1 ratio of bacteria and serum. The bacteria-serum mixture was plated onto nutrient broth agar in Petri dishes in triplicates. Samples were incubated at 0, 10, 20 and 30 min, for 37°C overnight. Negative controls consisted of 25% and 50% bacteria incubated with heated serum (56°C for an hour prior to experiment).

Each plate was inoculated with 50 µl of bacteria and serum mix onto a nutrient broth agar plate to determine the colony-forming units (CFUs) of each sample. After incubation at 37°C overnight, with the exception of *E. coli* which was grown for 48h due to slow growth. The colonies on each plate were counted using a manual colony counter.

Bacterial growth incubated in active sera for 10, 20 and 30 min were compared with growth at 0 min incubation, to determine the difference in bacterial growth as incubation times increased. The antibacterial capacity was determined by comparing the average bacterial growth of each bacterium in active sera at 10, 20 and 30 min incubation to bacterium in inactive sera at 10, 20 and 30 min incubation. All bacterial growths are measured using mean ±SD percentages of CFUs/mL.

6.4 RESULTS

Both red-tailed phascogale *Hp* and *CRP* cDNA sequences were confirmed using BLASTN [27]. The red-tailed phascogale Hp sequence obtained had an e-value of 3e-132 with nucleotide sequence identity of 292/308 (95%) with the Tasmanian devil Hp sequence (XM_003758541.1). The red-tailed phascogale *CRP* sequence obtained an e-value of 7e-31 with the sequence identity of 121/142 (85%) with the Tasmanian devil CRP sequence (XM_003767868.1). The comparison between protein sequences indicates that *Hp*, *CRP* and *SAA* marsupial sequences had protein identities above 60%. Protein alignments also included red-tailed phascogale *Hp* and *CRP*, and unrooted phylogenetic tree for *Hp*, *CRP* and *SAA* indicate that eutherian sequences were more similar to each other than marsupial sequences (Fig. 6.1). All protein alignments can be found in Supplementary Material 6.2-6.4.

Results indicate that *Hp* and *CRP* is expressed in all six red-tailed phascogale tissues examined; tongue, lung, heart, liver, gastrointestinal tract, spleen and kidney of adult and juveniles (Fig. 6.2). The presence of *Hp* was detected in red-tailed phascogales from ages three to 60 days, and the presence of *CRP* was detected from ages seven to 60 days (Fig. 6.2c), with BLASTN searches confirming sequence identities. The quantification of Hp in adult male and female red-tailed phascogale serum shows that concentrations of Hp are higher in males compared to females. Average haptoglobin protein levels obtained using the "PHASE" Haptoglobin Assay is presented in Table 6.1.

SAA concentrations in all red-tailed phascogale serum had an average of 4µg/mL (±0.277), with the lowest SAA concentration being 3.8µg/mL in one male juvenile and one adult female. SAA concentrations for all individuals were well below the 8 µg/mL cut-off level as defined by the SAA kit, presumably indicating that the red-tailed phascogale individuals were healthy (Table 6.1). However the horse serum utilised in the assay reported an SAA

concentration of 30µg/mL, indicating that the individual horse may have had a minor inflammatory disease at the time of sampling. For the antimicrobial analysis, serum diluted at 25% showed more effective results than serum diluted to 50%, and therefore were chosen for analysis, with a range of up to 300 CFUs present on the Petri dish for each bacterium. Compared to inactivated serum, results show that the growth of *K. pneumoniae* was reduced with time for male and female serum, showing an increase in percentage difference between bacteria introduced to active serum and inactivated serum (Fig. 6.3). While this decrease in bacterial growth is also seen with *E. coli*, *S. aureus* and *P. aeruginosa* for male and female serum from 10 to 20 min incubation times (except for male *P. aeruginosa* serum), an increase in bacterial growth was observed from 20 to 30 min incubation times. However, we still conclude that there is some bacterial protection against all the bacteria tested as positive percentage differences were observed at 20 and 30 min incubation times for *E. coli, S. aureus, P. aeruginosa* and *K. pneumoniae* (Fig. 6.3).

Even though the increase of CFU/mL is expected when bacteria is introduced to inactivated serum, there are still cases where the inactivated serum had lower CFU/mL than the average growth of bacteria introduced compared to active serum. Figure 6.4 shows the percentage difference between the two, and the negative percentage differences indicated that the CFU/mL of bacteria introduced to inactivated serum is higher than the average CFU/mL of bacteria introduced to active serum.

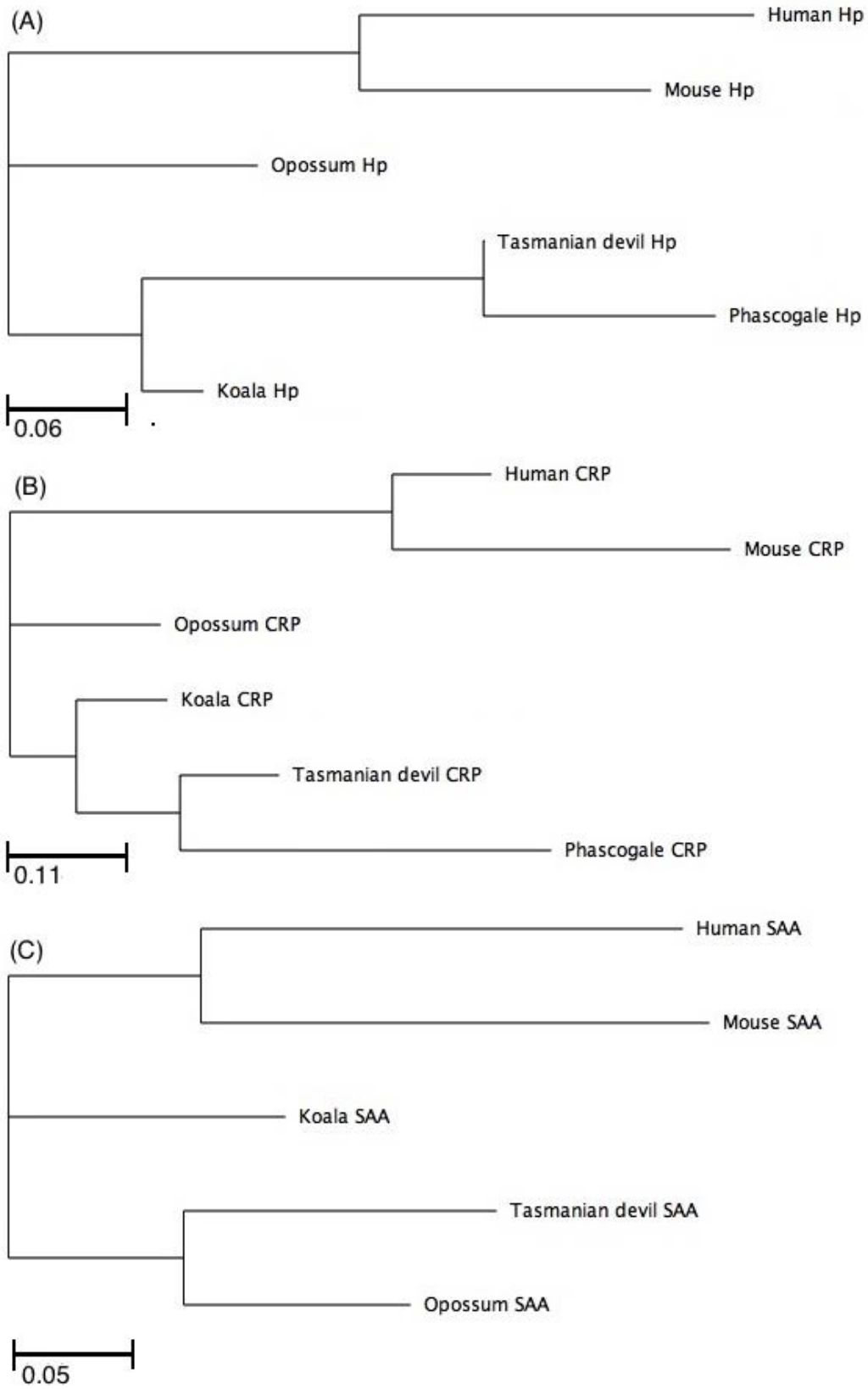

Figure 6.1 Unrooted morphological phylogenetic tree developed using **(A)** Hp, **(B)** CRP and **(C)** SAA protein sequences. Source of protein sequences can be found in Table 6.2.

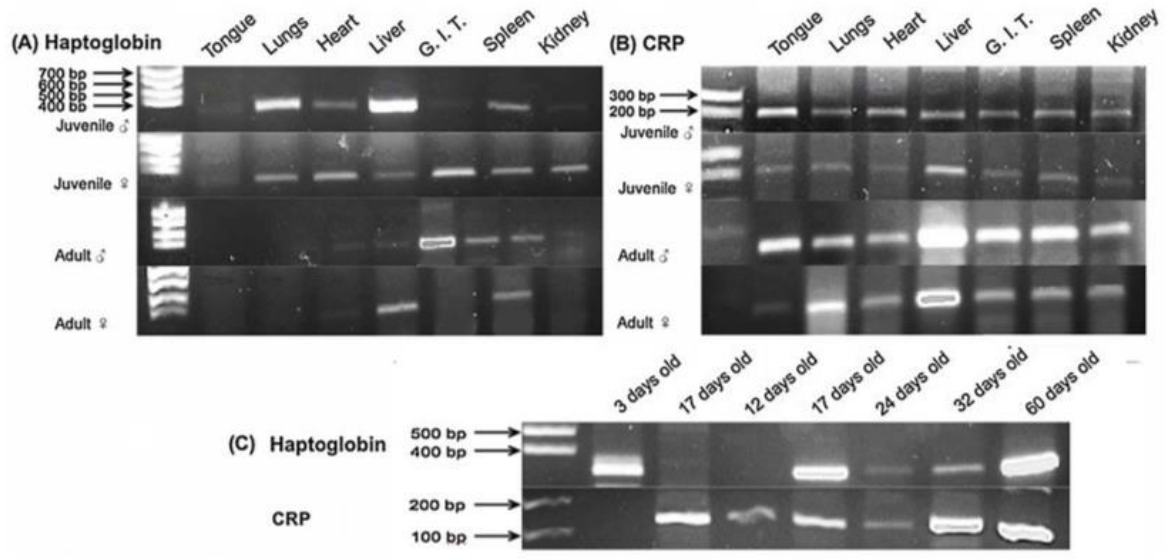

Figure 6.2 Kinetics of the antibacterial activities of male and female red-tailed phascogale, showing bactericidal properties of red-tailed phascogale serum against **(A)** *E. coli*, **(B)** *S. aureus*, **(C)** *P. aeruginosa* and **(D)** *K. pneumoniae* in comparison to results at 0t incubation. The results represent the means±SD for three replicates for each incubation time/bacterial introduction.

Table 6.1 SAA protein levels of nine individual red-tailed phascogales. Horse serum tested in resulted in an SAA protein level of 30 µg/mL.

Individual	Sex	Age	SAA concentration (µg/mL)
1	Male	5 months	3.80
2	Male	5 months	3.85
3	Male	5 months	3.83
4	Male	5 months	4.30
5	Male	5 months	4.50
6	Male	5 months	4.30
7	Female	3.25 years	3.85
8	Female	2.25 years	3.82
9	Female	5.25 years	3.80

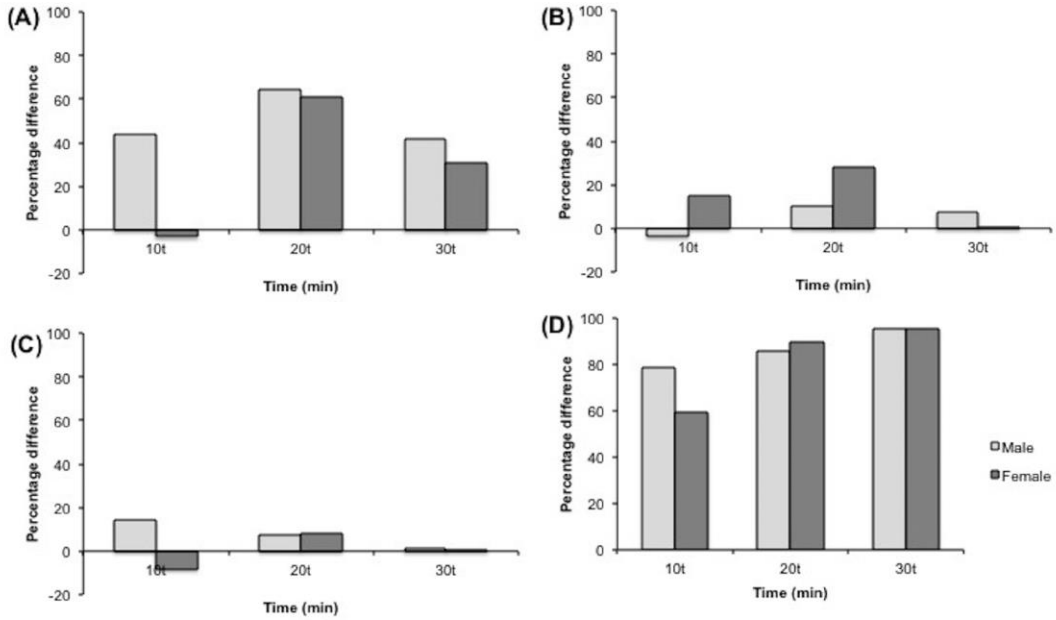

Figure 6.3 The percentage difference between the average CFU/mL of active and inactivated serum (heated at 56°C for 30 min) against **(A)** *E. coli*, **(B)** *S. aureus*, **(C)** *P. aeruginosa* and **(D)** *K. pneumoniae*. A positive percentage difference indicates that bacterial growth was higher when incubated with inactivated serum, which was expected prior to the experiment, as inactivated complement in serum would not have the ability to prevent bacterial growth.

6.5 DISCUSSION

In this study, we report the presence of APPs, Hp, CRP and SAA, in marsupial genomes, amplification of *Hp* and *CRP* genes in developing red-tailed phascogales using PCR, quantification of the levels of Hp and SAA proteins in juvenile males and adult females using commercially available assay kits, and found the capability of serum to provide some protection against three Gram-negative bacteria (*E. coli*, *P. aeruginosa* and *K. pneumoniae*) and one Gram-positive bacterium (*S. aureus*).

The identification of *Hp*, *CRP* and *SAA* sequences in marsupial genomes indicate that the APPs are present in marsupials. The protein alignments for *Hp*, *CRP* and *SAA* in eutherians (human and mouse) and other marsupial species reported identities above 60% (Table 6.2 and

6.3; Supplementary material 6.2-6.). The phylogenetic tree of *Hp*, *CRP* and *SAA* protein sequences show that the eutherian sequences (human and mouse) are in a clade, and so are red-tailed phascogale and Tasmanian devil sequences for Hp and CRP protein alignments. This result is not surprising given eutherian mammals are more closely related to each other, and red-tailed phascogales and Tasmanian devils are both dasyurid marsupials (Fig. 6.1).

Table 6.2 Average absorbance and concentration of haptoglobin in the male and female red-tailed phascogale, with rabbit and sheep serum as eutherian references.

Animal	Average Absorbance (630nm)	Haptoglobin protein levels (mg/mL)
Phascogale (male)	0.103	0.232
Phascogale (female)	0.120	0.410
Rabbit (sex unknown)	0.185	1.064
Sheep (sex unknown)	0.140	0.608

We identified the expression of *Hp* in pouch young as young as three days old through to adults using PCR, and quantified Hp in adult male and female red-tailed phascogale serum. The Hp level in serum is dependent on the species. For example, goat (*Capra aegagrus hircus*) Hp concentrations at 0.00 – 0.05mg/mL [33], and dog Hp concentrations of 0.3 – 3.5mg/mL are regarded as within the expected range for these species [34]. Rabbit and sheep serum in this study indicated Hp protein levels in these individuals were 1.064 and 0.608mg/mL, respectively. Male red-tailed phascogales had a higher Hp concentration compared to female red-tailed phascogales (Table 6.1). The normal range of Hp concentrations in marsupials, particularly red-tailed phascogales, have not been reported previously. The reason for higher Hp concentrations in males is unknown, but it is probable that high haptoglobin concentrations are related to male semelparity. Hp increases in mammals as a response to acute infection, inflammation, stress, neoplasia and trauma

(reviewed in [35]). For example, an increase in Hp levels was reported in goats with induced inflammation, with significant differences (P <0.05) occurring three and four days after inflammation was induced [33].

CRP is involved in the complement system and in mice has been reported to protect the body against microbial pathogens [36]. CRP has the ability to precipitate the somatic C-polysaccharide of *Streptococcus pneumoniae* [37]. Unlike Hp, the presence of CRP was not detected in the three day old pouch young, but in pouch young aged seven days to adulthood. Even though CRP was not amplified as early in newborn red-tailed phascogales, results obtained in this study still indicate pouch young are capable of responding to infectious agents, necrosis and trauma (reviewed in [38]) during relatively early postpartum life.

All red-tailed phascogale serum in this study had SAA concentrations <8µg/mL (Table 1), indicating that serum were likely extracted from healthy red-tailed phascogales. It has been reported that the *SAA* sequence in the tammar wallaby is different compared to its eutherian counterparts (i.e. human and mouse) due to concerted evolution [24], suggesting that *SAA* sequence similarity may only have occurred within a mammalian species, instead of between mammalian species. As we only tested the serum of presumably healthy red-tailed phascogale individuals in this assay, and had no clinically unhealthy individuals for comparison we cannot confirm that an SAA concentration of <8mg/mL confirms a healthy status in marsupials; however we can report that SAA concentrations of clinically healthy eutherians should report SAA concentrations <8µg/mL [39], and is presumably similar for red-tailed phascogales, assuming the function of SAA is similar in eutherians and marsupials. Although the horse serum was included as a reference to show that eutherian and marsupial SAA serum concentrations were comparable, the horse serum reported a unexpectedly high serum concentration of 30µg/mL, which may indicate the presence of a

minor inflammatory disease. According to Jacobsen *et al.* [39], clinically healthy horses and horses with non-inflammatory diseases usually reported SAA concentrations <0.48µg/mL while horses with inflammatory diseases reported an average of 1018µg/mL of SAA.

Based on previous studies we know that APPs are not the only immune molecules that have antimicrobial functions in marsupials. As stated previously the complement system is present and active in marsupial serum [9, 40], including the red-tailed phascogale [10]. While complement proteins have been reported to lyse foreign erythrocytes, proving that they have the capabilities to destroy foreign cells, the antimicrobial capacity of complement proteins and other serum components in the red-tailed phascogale are largely unknown. The results obtained in this study show the capability of serum to protect the body against three Gram-negative bacteria (*E. coli, P. aeruginosa* and *K. pneumoniae*) and one Gram-positive bacterium (*S. aureus*). Results indicated some growth inhibition was detected for all four bacteria tested when introduced to active serum. Overall, *K. pneumoniae* growth was most inhibited when introduced to red-tailed phascogale serum compared to inactivated serum, regardless of incubation time. The growth of *K. pneumoniae* was more than double the amount in inactivated serum compared to activated serum after 30 min incubation (Fig. 6.3). At the 30 min incubation time-point, bacterial growth inhibition was strongest against *K. pneumoniae*. The sensitivity of the type of bacteria to serum could depend on the structure and surface of the bacteria (reviewed in [41, 42]). As bacterium with lower sensitivity towards serum are more pathogenic to those that are not [42], results of this study indicate that *K. pneumoniae* likely has the highest pathogenic potential in red-tailed phascogales compared to *E. coli, S. aureus* and *P. aeruginosa.* In a study by Osawa et al. [43], *K. pneumoniae* was indicated as the casutive agent in the death of a pouch young koala, confirming *K. pneumoniae* is a pathogen of marsupials.

Table 6.3 Human and marsupial nucleotide sequences used, along with the E-value and identities when compared to their respective human sequences.

Gene	Human sequences	Marsupial sequences	E-values/Identities compared to human sequences
Haptoglobin (Hp)	NM_001126102.2	Opossum (XM_001378598.3)	E-value = 3e-173 Identities = 720/969 (74%)
		Tasmanian devil (XM_003758541.2)	E-value = 2e-150 Identities = 746/1042 (72%)
		Koala (Locus, 2585; Transcript, 13of73; Confidence, 0.015; Length, 1147)	E-value = 3e-114 Identities = 757/1018 (74%)
		Red-tailed phascogale	E-value = 2e-38 Identities = 203/285(71%)
C-reactive protein (CRP)	BC125135.1	Opossum (XM_007481666.2)	E-value = 1e-72 Identities = 483/696 (69%)
		Tasmanian devil (XM_003767868.1)	E-value = 2e-57 Identities = 426/619 (69%)
		Red-tailed phascogale	E-value = 0.001 Identities = 14/14(100%) *(In comparison to opossum nucleotide sequence, E-value = 7e-31; Identities = 121/142(85%))
Serum Amyloid A (SAA)	M26152.1	Opossum (XM_016423426.1)	E-value = 1e-37 Identities = 218/299 (73%)
		Tasmanian devil (XM_012552499.1)	E-value = 4e-38 Identities = 271/387 (70%)

*Red-tailed phascogale CRP nucleotide sequence was also compared to the gray-short tailed opossum sequence because of its high E-value. This result shows that red-tailed phascogale CRP have more sequence similarities to a marsupial compared to humans.

Table 6.4 Human and marsupial protein sequences used, along with amino acid identities when compared to their respective human sequences. Comparison is also made for translated red-tailed phascogale sequences.

Gene	Human sequences	Marsupial sequences	E-values/Identities compared to human sequences
Haptoglobin (Hp)	AAA88080.1	Opossum (translated from XM_001378598.3)	E-value = 0 Identities = 236/318 (74%)
		Tasmanian devil (XP_003758589.2)	E-value = 9e-178 Identities = 224/318(70%)
		Koala (m.132331 g.132331)	E-value = 1e-179 Identities = 235/318 (74%)
		Red-tailed phascogale	E-value = 3e-41 Identities = 55/88(63%)
C-reactive protein (CRP)	AAL48218.2	Opossum (translated from XM_007481666.2)	E-value = 2e-102 Identities = 140/224(63%)
		Tasmanian devil (translated from XM_003767868.1)	E-value = 2e-100 Identities = 135/225(60%)
		Koala (m.483439 g.483439)	E-value = 8e-99 Identities = 143/224 (64%),
		Red-tailed phascogale	E-value = 1e-10 Identities = 21/41(51%)
Serum Amyloid A (SAA)	AAA60297.1	Opossum (translated from XM_016423426.1)	E-value = 2e-48 Identities = 80/128(63%)
		Tasmanian devil (XP_003773620.1)	E-value = 4e-56 Identities = 80/126(63%)
		Koala (m.216752 g.216752)	E-value = 1e-53 Identities = 88/127 (69%)

Instead of high antimicrobial activity at the start of incubations, female serum against *E. coli* and, male and female serum against *K. pneumoniae* showed that their antimicrobial activity started off low but increased as time progressed from 10 to 30 min. The introduction of 25% (v/v) Komodo dragon serum against *E. coli* also showed a similar effect where CFUs decreased between 10 to 20 min incubation times [44]. The results of our study showed a steady decrease of *E. coli* growth for female serum but results were not significant ($P > 0.05$). Results were also not significant for male serum against *E. coli*; however bacterial activity was still detected for both male and female serum (Fig. 6.3).

As expected, the bacterial growth in inactivated serum resulted in a higher number of colonies compared to bacterial growth in activated serum for most samples. As inactivated serum is heated at 56°C for 30 min prior to the experiment, the heat should de-activate the serum by destroying cytolytic activity of the complement system, leading to deactivation of anti-bacterial proteins [45]. Samples that had higher bacterial growth in activated serum were all observed within the first 10 min of incubation, and applied to either male or female serum for all bacteria except for *K. pneumoniae*. Therefore, the results for active female serum introduced to *E.coli* and *P. aeruginosa* and active male serum introduced to *S. aureus* indicated that serum were not effective during the first 10 min of incubation (Fig. 6.3). Other results reported less bacterial growth when introduced to active serum compared to inactivated serum, showing the effectiveness of serum after 10 min.

The results of *E. coli* and *P. aeruginosa* after 10 min incubation could be dismissed due to the lower number of colonies in inactive compared to active serum. We also found that overall growth inhibition was higher for males than females for *E. coli* and *P. aeruginosa* after 30 min incubation, but higher for females compared to males for *S. aureus* and *K. pneumoniae*. The results suggest the capacity of individuals defending themselves against bacteria may be

reliant on gender, although the investigation of female pouches has indicated antimicrobial activity against *E. coli* and not *S. aureus* [46]. However, antibacterial activity against *S. aureus* and *K. pneumoniae* is usually passed on from mother to offspring during lactation [47, 48]. Further investigations are required to assess differences in male and female immune competence in marsupials.

The results of this study confirm the presence of APPs; Hp, CRP and SAA, and that marsupial serum has antimicrobial propertiesthat can inhibit some Gram-positive and Gram-negative bacterial growth. The results of this study provide evidence that suggest marsupials have robust innate immune mechanisms, particularly with respect to their capacity to deal with early immune challenges. This study provides the basis for further studies on APPs in marsupials and the innate immune mechanisms, particularly newborn marsupials.

6.6 CHAPTER 6 REFERENCES

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6.7 CHAPTER 6 SUPPLEMENTARY MATERIALS

Additional Supporting Information may be found in the appendix:

Supplementary Material 6.1 The sexes, ages, and tissues extracted from individual red-tailed phascogales (*Phascogale calura*) for the identification of haptoglobin (Hp) and C-reactive protein (CRP).

Supplementary Material 6.2 Protein alignment of human (GenBank: AAA88080.1), mouse (GenBank: AAB29697.2), gray short-tailed opossum (translated from nucleotide GenBank: XM_001378598.3), Tasmanian devil (GenBank: XP_003758589.2), koala (Koala Genome Consortium: m.132331 g.132331) and red-tailed phascogale haptoglobin (Hp) sequences.

Supplementary Material 6.3 Protein alignment of human (GenBank: AAL48218.2), mouse (GenBank: CAA31928.1), gray short-tailed opossum (translated from GenBank: XM_007481666.2), Tasmanian devil (translated from GenBank: XM_003767868.1), koala (Koala Genome Consortium: m.483439 g.483439) and red-tailed phascogale C-Reactive Protein (CRP) sequences.

Supplementary Material 6.4 Protein alignment of human (GenBank: AAA60297.1), mouse (GenBank: NP_033143.1), gray short-tailed opossum (translated from XM_016423426.1), Tasmanian devil (GenBank: XP_003773620.1), koala (Koala Genome Consortium: m.216752 g.216752) and red-tailed phascogale Serum Amyloid A (SAA) sequences.

CHAPTER SEVEN

CONCLUDING SUMMARY AND FUTURE DIRECTIONS

7.1 CONCLUDING SUMMARY

Immune systems play an important role in species survival, which is why the study of immunology is particularly important for marsupials. A few marsupial species, including the red-tailed phascogale, are experiencing population declines, whether it is due to disease or sensitivity towards the fluctuating environment. So far, the general knowledge of immunity (Janeway et al. 2001), along with mammalian immunology/genetic studies have contributed to our understanding of infections and disorders in marsupials, and thus contributed to the prevention and management of diseases (Miller & Herbert 2010; Siddle et al. 2010; Warburton 2014). However, more information about the marsupial immune system is still needed to understand the development of the marsupial immune system, the successful survival of underdeveloped marsupial young, and the diseases associated with marsupial immunology.

Altogether, the results obtained in this thesis have successfully identified complement and complement-related components in non-eutherian mammals, investigated the expression of major key complement factors in marsupial pouch young, juveniles and adults, and confirmed a functional complement system in a marsupial and its potential antimicrobial activities. In addition, there is now evidence that alongside opossums and the tammar wallaby, red-tailed phascogales can also be useful models for comparative immunological studies.

As a preliminary study, the presence of complement and complement-related sequences were confirmed in the genomes of four marsupials and one monotreme; the gray short-tailed opossum, tammar wallaby, Tasmanian devil, koala and platypus respectively. Even though the research was constrained by the quality of the genome, it was enough to demonstrate that non-eutherian mammals have a comparable complement system and complement-related

components to humans and other eutherian mammals, with highly conserved sequences identified between the mammalian groups. Motif comparisons indicate that major complement components, including C3, C1q, CFP and MASPs have similar function to that of eutherians, and a phylogenetic tree using C3 sequences in non-eutherian mammals suggest that complement components are orthologous to eutherians, but are divergent between the three mammalian groups. The similarities between eutherian and non-eutherian sequences suggest the presence of a complement system and the possibility of the use of complement system in non-eutherians.

The identification of these complement sequences reinforced the idea that the production and function of serum proteins are seemingly similar in mammalian groups. Using this information, alongside with the knowledge of similarities between non-eutherian and eutherian sequences, future complement studies can model investigations of marsupial immunity on similar studies with confidence, especially since eutherian complement studies have been well-established. While the identification of complement sequences in non-eutherian genomes is important, quantitative studies are also informative as comparisons can be made on a numerical/reproducible basis in qPCR studies. Studies of gene expression in marsupials can only be experimentally meaningful if the reference genes chosen are stable enough in the type of tissue and experimental conditions (reviewed in Morris, Wong & Belov 2010).

In this thesis, the stability of *GAPDH*, *ACTB*, *18S rRNA*, *28S rRNA*, and *RPL13A* of liver, lung, small intestine and spleen tissue samples in two age groups was found. Differences were identified in the stability of the reference genes according to the type of tissue and age group, information that should be considered in other marsupial expression studies, as it suggests that there is no one reference gene suitable for a variety of tissues and

age groups. The selection of a stable reference would allow for appropriate normalisation of RNA or cDNA in experiments with different volumes of starting material, and dissimilarities in RNA preparation and cDNA synthesis (Radonić et al. 2004).

The expression of reference genes in two age groups combined could be applied to developmental studies, where the expression of samples across various ages is needed. In separating the results for juvenile and adult expression, the study indicated that suitable reference genes may differ, showing that the expression of the same reference gene in different age groups could indicate differences in gene stability. The results presented could be useful to future studies for selecting the most suitable and stable reference genes, especially if analysing expression in tissues related to immunological development, immunological responses and complement production (Haviland et al. 1995; Drouin et al. 2001; Wang et al. 2010). The use of two reference genes is also needed for proper normalisation of gene expression studies (Bustin et al. 2009), and the results provided in this thesis is able to provide stability information for more than one reference gene, allowing more robust comparisons between studies of gene expression in similar tissues.

Further studies were employed to give an insight into the development of a young marsupial's complement system. To study the expression of complement proteins in developing red-tailed phascogales would provide an indication as to whether the complement system is functional in all age groups, and may suggest a further method newborn marsupials utilise to protect themselves in the microbial-rich environment they enter into when they lack mature immune tissues. The sequence identification of C3 and MASP2 molecules in adults were first investigated, and partial cDNA sequences were obtained for the two complement components. The comparison between the translated protein sequences of C3 and MASP2 in the red-tailed phascogale, and their respective eutherian sequences confirmed conserved

sequence structures, with the identification of important protein function components relating to the complement system. The translated C3 sequence has also led to the identification of C3a in the C3 sequence, an anaphylatoxin that is able to suppress bacterial infection in humans (Nordahl et al. 2004).

The successful amplification and expression of MASP2 also provided important information regarding a functional Lectin Complement pathway. A way to detect a functional Lectin Complement pathway is to obtain anti-C1q antibodies for the species in question, to avoid the activation of the Classical Complement pathway due to similarities in the two pathways (Roos et al. 2003). The amplification and expression of MASP2 suggests the utilisation of the MASP2 protein in complement activation and hints at the possibility of a functional Lectin Complement pathway in marsupials.

To complete the research presented in this thesis, another two complement proteins, C1r and CFP, were selected for complement expression because of their importance in the remaining two complement pathways, Classical and Alternative, respectively. The results of this portion of the study found that complement expression relative to reference genes differed among the different age groups, but all four complement components were expressed in pouch young, juvenile and adult red-tailed phascogales. From this study, we have confirmed the continuing maturation of the red-tailed phascogale immune system, as pouch young grew older, with the lowest complement expression occurring in day one post-partum pouch young. The finding that pouch young express complement proteins is particularly interesting as marsupial pouch young presumably depend on innate and maternally-derived immunity during early postnatal life, and are not known to have a functional adaptive immune system at birth (reviewed in Old & Deane 2000). However, the expression of major complement components, including C1r from the antibody-dependent Classical Complement

pathway, suggests the capacity for the classical complement pathway to be used as a defence mechanism, and suggesting that complement proteins may be passed on from mother to pouch young via milk. Unfortunately, we could not detect the main production site of complement proteins in marsupial young, if there is one, as their livers were too small to extract, leaving whole body tissue samples as the only option for the expression study.

Liver tissue samples were used for juvenile and adult males because dissection was possible in larger individuals. Results indicate that there were higher complement expression levels in juveniles compared to adults. The reason for this decrease in expression is uncertain, however there are other aspects of the study that could be explored in future studies. Adult male red-tailed phascogales in this study had been through a breeding season, therefore there is the possibility that lower expression levels are linked to a possible immunosuppression that occurs after the first breeding season. As the study was performed on individuals that were opportunistic, there were no female samples to compare these results to, which would have proven interesting as it could potentially reinforce the differences between females and males after the first breeding season.

Even though we did not directly identify a functional Lectin Complement pathway in a marsupial, we managed to identify functional Classical and Alternative Complement pathways in red-tailed phascogales. A general method has been written to identify the Classical and Alternative Complement pathway by using sheep and rabbit erythrocytes, respectively (Morgan 2000). This methodology not only applies to eutherians, but according to previous marsupial studies (Wirtz & Westfall 1967; Koppenheffer, Spong & Falvo 1998), can be used to detect functional complement systems in marsupials, making results comparable to other functional complement studies including non-eutherian (Wirtz &

Westfall 1967; Koppenheffer, Spong & Falvo 1998) or non-mammalian studies (Merchant et al. 2006).

A functional complement system was detected in red-tailed phascogales by testing complement activity in serum. As the methodologies were similar to that used in opossum studies (Wirtz & Westfall 1967; Koppenheffer, Spong & Falvo 1998), the functional Classical Complement system identified in red-tailed phascogales were not only comparable to eutherians but also other marsupials. Results indicate that sensitised sheep erythrocyte lysis occurred at similar temperatures to opossums, which is not surprising as both opossums and red-tailed phascogales presumably have comparable immune systems. Some male Virginia opossums have even been reported to go through immunological/physiological changes similar to semelparous males (Woods II & Hellgren 2003). Similarly, the presence of a functional Alternative Complement pathway was also detected in red-tailed phascogale using rabbit erythrocytes, as reported in opossums (Wirtz & Westfall 1967; Koppenheffer, Spong & Falvo 1998).

Prior to these experiments, the differences in complement function between male and female marsupials had not been investigated. The results in this study have suggested female phascogale serum is more effective at eliminating foreign cells compared to male phascogale serum. Similar to the decreasing complement expression study, this result could possibly be due to male semelparity. We now know that both Classical and Alternative Complement pathways are functional in red-tailed phascogales, adding to the information provided by Wirtz and Westfall (1967) and Koppenheffer, Spong and Falvo (1998) on the function of the complement system of marsupials.

Complement activation leads to the generation of antimicrobial peptides (Nordahl et al. 2004), which work with other serum proteins to inhibit bacterial growth. In this thesis, the

antibacterial effectiveness of phascogale serum against four different bacteria was investigated. Results indicate that red-tailed phascogale serum suppressed the growth of three Gram-negative and one Gram-positive bacteria. After identifying the presence of a functional complement system in red-tailed phascogale, the results provide further support that serum proteins in marsupials are comparable to eutherians. While antimicrobial properties of eutherian serum has already been established (Reller & Stratton 1977), antimicrobial activities in marsupials have only been reported in the pouch (Bobek & Deane 2001; Ambatipudi et al. 2008) or maternal milk (Wanyonyi et al. 2011) for the protection of young, but not in serum.

It is known that the complement system is not the only immune component contributing to the elimination of pathogens or bacterial growth inhibition, therefore positive APPs that are involved in the innate immune system were also investigated. From the results in this thesis, we know that APPs, Hp, CRP and SAA, are all present in red-tailed phascogales. Hp and SAA were expressed at relatively low concentrations as serum was extracted from healthy red-tailed phascogales. From other mammalian studies, it has been reported that Hp concentrations only increase after the innate recognition of foreign cells (Couper et al. 2005; Scudellari 2009), however serum of sick individuals were not tested in this thesis as samples were opportunistic. The results also indicated that *Hp* and *CRP* were amplified in developing red-tailed phascogales, showing the presence of Hp and CRP in red-tailed phascogales ≤7 days old. In eutherians, it has been reported that APPs are functional in newborns (Short & Hide 2015). Various APPs, including CRP, are expressed at low levels at birth but increase dramatically during the first week of life (reviewed in Hasegawa et al. 2014), however this has not been reported for marsupial young. Currently, there are limited marsupial APP studies, but with the results obtained in this thesis, it provides a basis for

future studies that aim to distinguish healthy from unhealthy animals based on the volume of specific APPs that are present in serum.

The results of this thesis demonstrate that the complement system is present and functional in non-eutherian mammals, including the red-tailed phascogale. We now know of the existence of the complement system in a dasyurid species, and their ability to use complement as a form of immunological protection. The experiments in this thesis have therefore successfully added to the scarce knowledge of the marsupial complement system.

7.2 Future Directions

The results presented in this study confirm the presence of complement components in non-eutherian mammals, and the expression of functional complement proteins in red-tailed phascogales in three different age groups. These studies present an opportunity to pursue future research on the complement system of marsupials. Since this thesis has shown the presence of a functional complement system in marsupials, we now need to focus research on more specific aspects of the survival of marsupial pouch young and the development of complement as they mature into adults.

Prior to this work, there were only a limited number of marsupial complement studies, and there were no complement studies conducted on dasyurids. Since the haemolytic assay has now been optimised for use in the red-tailed phascogale, a dasyurid, it can now be performed on other marsupial species, and comparisons made in animals with and without disease. Further, as haemolytic assays have been used to detect functional complement systems in reptiles (Merchant et al. 2006; Bourin et al. 2013) and fishes (Sakai 1992; Holland & Lambris 2002), investigations of the complement system in various marsupial species was long overdue. As we found that female red-tailed phascogale serum was better at lysing sheep

and rabbit erythrocytes compared to male serum, and that complement expression in males decreased after their first breeding season, future studies should consider investigating the link between male semelparity and complement function. Even though it has been reported that concentrations of plasma cortisol and plasma albumin did not change in captive males compared to free living males after their first breeding season in phascogales (Schmidt et al. 2006), it is still possible that there is a link between semelparity and complement expression as captive males are reproductively senescent (Bradley 1997).

Previous studies have reported the susceptibility of dasyurids to various diseases. Captive dasyurids, in particular, live longer than free living dasyurids and therefore have been linked to several age-related diseases in senescent individuals (Holz & Little 1995). Even though they are not particularly old, compared to female phascogales, captive males are no longer able to reproduce after the first year of their lives, making male phascogales more susceptible to diseases in addition to being reproductively senescent (Bradley 1987; Schmidt et al. 2006). As this study has indicated the possible link between the complement system and semelparity, more comparisons between male and female complement systems, and the immune system in general, are needed to reinforce the connection between the two factors.

As this thesis is first to report the expression of complement proteins in developing marsupials, methodologies used in this thesis can be used for other complement expression studies on marsupials, and the results can be used for comparative studies in complement expression studies within this mammalian Order. In terms of limitations, even though the number of samples in this study has been sufficient to conduct various experiments, the samples obtained were opportunistic, and therefore limited the number of individuals able to be utilised in the current study. In addition, to investigate the complement expression in developing red-tailed phascogales proved to be difficult due to the body size of pouch young.

Therefore, we suggest that laboratories with opportunities to perform dissections on very small tissue samples, or source larger marsupials, should consider conducting a complement expression study so the statistical comparison between marsupial young and adults can be made. With constant improvements in qPCR methods, more in-depth expression studies are becoming more and more feasible. Confirmation of the main complement production site would be useful as future studies would then be able to focus on those particular tissues, and determine if complement plays a more specific role in pouch young immunity.

Isolation of more marsupial complement sequences would also be useful. As experiments in this study have managed to isolate two major complement components, C3 and MASP2, and determine their expression in developing marsupials, future studies now have the opportunity to investigate the expression of other complement proteins such as C1q, a protein largely involved in the Classical Complement pathway. Subsequently, further research could either reinforce the use of complement proteins in marsupials and eutherians, or identify differences in the two mammalian groups. Single cell analyses are also encouraged, as they can be performed to analyse multiple gene expression in a single cell rather than whole tissues, and there is opportunity to analyse different expression profiles of individual cells in a particular tissue (Scudellari 2009).

As sequences have already been identified in five non-eutherian mammal genomes, it is now possible to use that information to isolate more complement sequences in other non-eutherian mammals, and investigate the differences and similarities between the function and expression of these genes. Complement regulators and inhibitors were limited to identification in non-eutherian mammal genomes in this thesis, but are likely just as important as complement proteins involved in the three main complement pathways of marsupials. Complement regulators in eutherians have been linked to various diseases,

including glomerulonephritis (Morita et al. 1997) and hemolytic uremic syndrome (Richards et al. 2003). It is possible if complement protein structure is similar in marsupials, to that of other mammals, that complement protein regulation is also comparable, however further studies would be required to confirm these suggestions.

Dasyurids are highly susceptible to *Toxoplasma gondii*, a parasitic infection (Finnie 1988). Fuhrman and Joiner (1989) reported that *T. gondii* affected the formation of MAC, making it complement-resistant. However, a later study found that complement activation upon the invasion of *T. gondii* depends largely on the type of antibody activating the complement system (Couper et al. 2005), reinforcing the importance of IgM and IgG to the complement system. Bacterial infections, such as salmonellosis, also affect dasyurid populations with *Salmonella spp.* isolated from scats of free living dasyurids (reviewed in Obendorf 1993). Research has found that dasyurids are susceptible to *Salmonella* serotypes and *Mycobacterium* species (Johnson et al. 2014), leading researchers to suggest the maintenance of *MHC* diversity (Cheng et al. 2012). The *MHC* region encodes for complement proteins, and studies conducted in this thesis have shown that complement is likely functional in a marsupial. The susceptibility of dasyurids towards infections suggests that further investigations of the complement system, or complement-linked immune components, would be useful to increase our understanding on the marsupial immune system and potentially aid in the conservation of declining dasyurid species.

7.3 CHAPTER 7 REFERENCES

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ADDITIONAL PUBLICATIONS

HEALTH AND DISEASE IN FREE-RANGING EASTERN GREY KANGAROOS (*MACROPUS GIGANTEUS*)

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ABSTRACT

Baseline hematology, blood chemistry and acute phase protein parameters have not previously been published for free-ranging eastern grey kangaroos (*Macropus giganteus*). Eight eastern grey kangaroos including three adult males, three adult female and two sub-adult males from two different populations were examined. Differences relating to age and gender were detected in both the hematological and blood chemistry values. Assays assessed the antibacterial activity of kangaroo serum against three Gram-negative, and one Gram-positive bacteria. The kangaroo serum had a strong antibacterial response *to Klebsiella pneumoniae*, and moderate responses to *Escherichia coli* and *Staphylococcus aureus*. The presence and concentration of the acute phase proteins, haptoglobin and serum amyloid A in kangaroos was investigated. Haptoglobin and serum amyloid A were present in kangaroo serum, but only haptoglobin was elevated in a kangaroo with capture myopathy and necrotic wounds. The findings of this study provide preliminary data on health parameters of free-ranging eastern grey kangaroos. These parameters can be used to assist in assessing health in free-ranging populations.

Keywords: macropod; marsupial; hematology; blood chemistry; antibacterial defense; acute phase protein

1. INTRODUCTION

Eastern grey kangaroos (*Macropus giganteus*) are an abundant, iconic and easily recognizable Australian species; however there is a lack of available data on health and disease for the species. Generally, there is limited information available on the hematological and blood chemistry values of macropod species, especially for wild macropods. Hematology reference intervals have been determined for some free-ranging macropod species (Arnold, 1987; Arundel et al., 1979; Barnes et al., 2008; Robert and Schwanz, 2013; Ruykys et al., 2012; Shield, 1971; Spencer and Speare, 1992; Stirrat, 2003; Vaughan et al., 2009). Blood chemistry and hematology values have been published for a single eastern grey kangaroo infected with gamma-herpesvirus (Wilcox et al., 2011), and some limited hematological data for free-ranging eastern grey kangaroos (n= 14 yearlings, 10 adults), and red kangaroos (*Macropus rufus*) (n=57) at different stages of maturity. However this study only conducted total white blood cell counts and no differential white blood cell count data was included (Presidente, 1978).

Eastern grey kangaroos generally exhibit high stress levels making them susceptible to a range of diseases (Staker, 2006), such as bacterial infections from *Escherichia coli* and *Leptospira weilii* (Gordon and Cowling, 2003; Roberts et al., 2010b), viruses such as *Herpesvirus* (Wilcox et al., 2011), as well as parasites including *Cryptosporidium* (Power et al., 2004), multiple species of Coccidia (Staker, 2006), and over 20 species of strongyle nematodes (Garnick et al., 2010). It is likely that the high disease prevalence in this species is attributed to their large, high density, open membership groups, and close foraging proximity which enables frequent transmission between individuals (Cripps et al., 2016).

Serum contains antimicrobial properties such as acute phase proteins (APPs) and complement proteins (Cray et al., 2009; Sarma and Ward, 2011). A key response during

inflammation or infection in either humans or animals is a drastic increase in the concentration of acute phase proteins, including haptoglobin (Hp) and serum amyloid A (SAA) (Cray et al., 2009). The biological function of Hp is to bind to hemoglobin in order to prevent excessive loss of iron through urinary excretion as Hp helps in regulating the renal threshold for hemoglobin (Putnam, 1973). The levels of Hp increase during infection, inflammation, trauma or disease. However Hp decreases during severe hepato-cellular deficiency and hemolytic conditions (Dobryszycka, 1997).

SAA is a major acute phase protein that has been identified in multiple mammal species including humans (*Homo sapiens*) (Whitehead et al., 1992), mice (*Mus musculus*) (De Beer et al., 1994), and horses (*Equus caballus*) (Jacobsen et al., 2006). SAA is an indicator of acute inflammation however the slower reacting Hp indicates chronic inflammation (Alsemgeest et al., 1994; Horadagoda et al., 1999). Horses with inflammation or tissue damage have been found to have higher SAA concentrations than healthy horses. Measuring SAA levels may be a useful method of monitoring disease activity in mammals (Hulten and Demmers, 2002). Both Hp and SAA presence was assessed in eastern grey kangaroos to evaluate whether marsupials, like eutherians, are able to produce acute phase proteins, triggered during an infection or trauma.

The overall objective of this study was to determine measures of health in free-ranging eastern grey kangaroos by 1. Determining baseline blood chemistry and hematology levels, 2. Verifying the presence and levels of acute phase proteins, and 3. Examining the antibacterial response of serum.

2. METHOD

2.1. STUDY SITES

Data was collected at two sites in NSW (Figure 1). One site was a semi-rural site surrounded by built infrastructure such as roads and buildings. The other site had relatively little built infrastructure and is a conservation area surrounded by National Parks land containing cliffs and bushland.

Yarramundi Paddocks, Hawkesbury Campus, Western Sydney University NSW (33°36'591"S/ 150°43'706"E) is located on the corner of Bourke Street and Londonderry Road Richmond, NSW. The 'Yarramundi Paddocks' has an area of approximately 308 ha, and consists of pastures, grasslands, marshes, and open woodland.

The Hawkesbury campus is located in a semi-rural area and is surrounded by urban development. Built infrastructures create barriers which may limit the directions in which animals can disperse, and reduce areas suitable for foraging which is likely to effect the dispersal of animals in this area.

The Wolgan Valley Resort and Spa, NSW (33°15'08.60"S/ 150°11'597"E) is located in the Wolgan Valley, NSW, is a 1619 ha property surrounded by National Parks and sandstone cliffs. There is significantly less built infrastructure, and therefore fewer barriers in this area than at the campus site. Data from the campus sites and the Wolgan valley conservation area were compared to evaluate differences between eastern grey kangaroo populations in these areas.

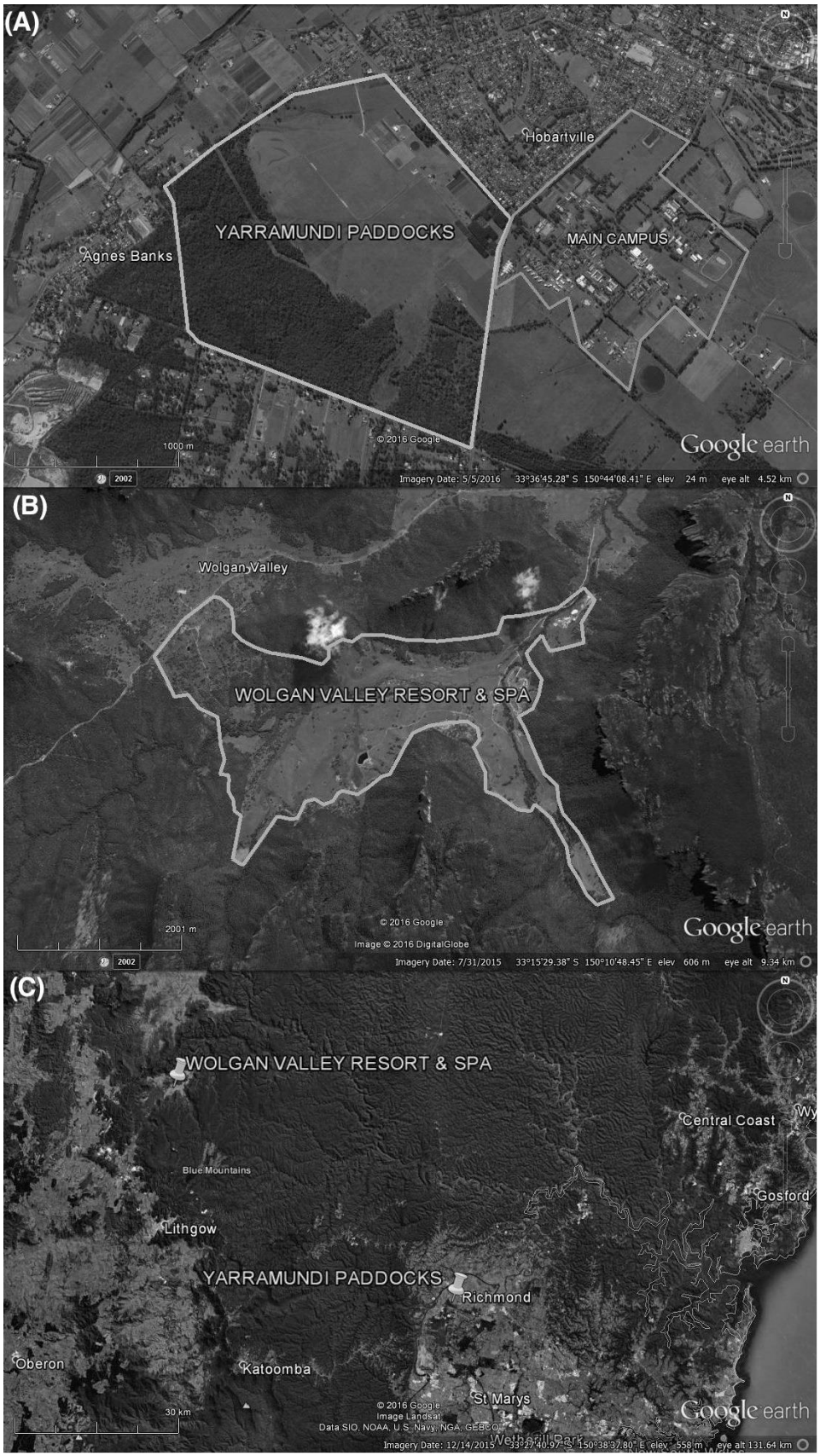

Fig. 1. Data collection sites: (A) Hawkesbury campus site, (B)Wolgan valley site, and (C) The relative locations of the Hawkesbury campus and Wolgan valley sites in NSW.

2.2. *Animals*

A total of eight wild eastern grey kangaroos were sampled. There were three adult males captured at 'Yarramundi Paddocks', and three adult females captured at the Wolgan Valley. Two sub-adult males were captured, one at 'Yarramundi Paddocks', and one at the Wolgan Valley. All animals were captured from June to October 2014 and 2015 (late winter to spring). One adult male was euthanized by a veterinarian after capture due to capture myopathy (unpublished result).

2.3. *Field procedures*

There is a need to obtain hematology and blood chemistry data from wild macropods to increase available data on clinical health assessment. However, utilizing wild macropods has potential safety risks (King et al., 2011). The size and strength of kangaroos are a risk to the researcher, and there is a high risk of injury to the animal when using traps or nets to capture macropods (King et al., 2011). For the safety of researchers and kangaroos it is necessary to anaesthetize animals prior to capture to minimize some of the risks associated with the capture and handling of wild macropods.

Kangaroos were darted in the upper hind leg, thigh or rump from a distance of 23-28 m in accordance with recommendations made by Roberts et al. (2010a). Animals were chemically restrained using tranquilizer darts containing 5-7 mg/kg of Zoletil 100, fired from a CO_2 Injection Rifle (Dan-Inject, Denmark). When the kangaroo dropped to the ground they were approached slowly and quietly, a hessian sack was placed over their head and the tail and hind limbs were restrained during processing.

Morphometric measurements were collected from each individual including foot length (with and without toenail), tail diameter, tail length and total body mass. Body condition was estimated from morphological measurements. Fur condition, the presence of injuries, and evidence of ectoparasites were also recorded.

2.4. HEMATOLOGY AND BLOOD CHEMISTRY

A blood sample was taken from the tail vein using a 21 gauge needle from all kangaroos captured. Two thirds of the sample was placed immediately into an EDTA coated Vacutainer tube (Becton, Dickinson and Company, New Jersey) and the remaining third into a Venosafe tube containing a clot activating additive (Terumo Europe, Belgium) before transportation to the laboratory.

Blood chemistry values were measured using a Vetscan VS2 blood chemistry analyzer and comprehensive diagnostic profile rotor plates (Abaxis, Union City, USA). The rotor plates measured the following parameters; albumin (g/L), alkaline phosphatase (U/L), alanine aminotransferase (U/L), amylase (U/L), urea nitrogen (mmol/L), creatinine (μmol/L), globulin (g/L), glucose (mmol/L), potassium (mmol/L), sodium (mmol/L), phosphorus (mmol/L), total bilirubin (μmol/L), and total protein (g/L). White blood cell counts were calculated from manual counts of blood smears on a microscope slide stained with DiffQuick stain (Bacto Laboratories Pty Ltd, Mount Pritchard, Australia) using an Olympus CX31RBSF compound microscope.

2.5. EVALUATION OF ANTIBACTERIAL ACTIVITY

An antimicrobial assay was performed to assess the antibacterial activity of kangaroo serum. Serum was pooled from multiple individuals for each gender. Pooled serum was then

diluted to make concentrations of 25% and 50% serum using a sterile saline solution. Heated serum (56 °C for 30min with shaking), and saline were used as negative controls for comparison.

Three Gram-negative bacteria; *Escherichia coli (K12), Pseudomonas aeroginosa (ATCC 27853), Klebsiella pneumoniae (ATCC 13883*), and one Gram-positive bacteria; *Staphylococcus aureus (ATCC 25923),* were used in this study. Bacteria were grown on nutrient agar slants and transferred to a sterile nutrient broth liquid culture. The culture was incubated overnight to obtain a log-phase in a 37 °C shaking incubator. The optical density of the culture was tested using the Benchmark Plus™ microplate spectrophotometer (Biorad, Hercules, USA) at a 600nm wavelength to ensure the optical density was between 0.2-1 (growth phase). The culture was then diluted to a concentration of 1x10^5 with sterile 0.9% saline.

Equal volumes of diluted bacteria were added to the diluted serum, heated serum, and saline to create a 1:1 ratio. Serum-bacterial solutions were incubated for increments of 0, 10, 20 and 30 min at 37 °C with shaking. Solutions were then plated in triplicate (50 µl each) on nutrient agar plates immediately following each incubation time. Inverted plates were incubated overnight (37 °C). Colonies were counted and used to obtain averages. Colony forming units (CFUs/mL) was calculated using the method described in Merchant (2006) (colonies X dilution X10).

2.6. Acute Phase Protein Assays

2.6.1. Haptoglobin Assay

The PHASE Haptoglobin assay (Tridelta Development Ltd, Bray, Ireland) was used according to the manufacturer's instructions to determine the presence and concentration of

Hp protein in eastern grey kangaroo serum, using rabbit (*Oryctolagus cuniculus*) and sheep (*Ovis aries*) serum as comparative controls. Rabbit serum was supplied by Sigma-Aldrich Pty. Ltd. (Castle Hill, Australia), and sheep serum was supplied by Serum Australis Pty. Ltd. (Manilla, Australia). Serum from two male kangaroos (one healthy and one unhealthy with evidence of necrotic wounds and capture myopathy) were utilized. A set of Hp standards with known concentrations (2.5mg/ml, 1.25mg/ml, 0.625mg/ml, 0.312mg/ml and 0mg/ml), and serum samples were plated in triplicate in a microtitre plate. The absorbance was read at 630nm using a Benchmark Plus™ microplate spectrophotometer, and the results interpreted based on a standard curve of known Hp samples.

2.6.2. SERUM AMYLOID A ASSAY

The LZ Test 'Eiken' SAA kit (Eiken Chemical Co., Ltd ., Tokyo, Japan) was used to determine SAA protein concentration in the serum from three adult male eastern grey kangaroos (two healthy, and one unhealthy that showed signs of capture myopathy and necrotic wounds), as well as three adult female and two sub-adult male eastern grey kangaroos. Horse serum was also used as a eutherian reference for comparison. Horse serum was supplied by Vector Laboratories (Burlingame, USA). This serum was collected from healthy horses and heat treated at 56°C for 2 hours, stored at 4°C for several weeks, and centrifuged through a 0.45µm filter to remove precipitates. The test was performed according to the manufacturer's instructions using a 96 well microplate, and analyzed using a Benchmark Plus™ microplate spectrophotometer.

3. RESULTS

All kangaroos in this study recovered from anesthesia without ill effects, with the exception of one adult male which exhibited signs of capture myopathy and was euthanized as a result. All study subjects (with the exception of the euthanized male) appeared to be in good health with adequate body condition, no evidence of injuries, and no obvious ectoparasites. Adult males (n=3) had a mean weight of 43 kg (± 3.62) and adult females (n=3) had a mean weight of 27 kg (± 6.36). The sub-adult males (n=2) had a mean weight of 18 kg (± 2.33). The foot length of adult males was 36 cm (± 0.87), and including the longest toenail was 39 cm (± 1.26), the foot length of adult females was 30 cm (± 1.26), and including the longest toenail was 33 cm (± 1.53), and the foot length of the sub-adult males was 31 cm (± 0.07), and including the longest toenail was 33 cm (± 0.07). The tail of adult males was 107 cm (± 5.51) long with a diameter of 89 mm (± 22.59), the tail of adult females was 78 cm (± 5.41) long with a diameter of 42 mm (± 12.16), and the tail of the sub-adult males were 71 cm (± 2.83) long with a diameter of 54 mm (± 22.37). All three adult females were lactating and had pouch young. Two females also were observed to have young at foot.

3.1. BLOOD CHEMISTRY

Notable differences in blood chemistry (Table 1) were observed between genders and between adults and sub-adults. Mean levels of alkaline phosphatase were higher in female eastern grey kangaroos (341 U/L) than sub-adults (145 U/L) and adult males (97 U/L). Adult females had lower levels of alanine aminotransferase (53.67 U/L), than both the adult males (77 U/L) and sub-adults (66 U/L). Amylase was found to be higher in sub-adults (282 U/L). Adult males had distinctly higher levels of creatinine (125 μmol/L) and glucose (8 mmol/L).

Table 1. Blood chemistry of eastern grey kangaroos from two populations from two populations in NSW during winter and spring 2014.

Blood chemistry	Male (n=2)		Female (n=3)		Sub-adult male (n=2)	
parameters	mean	SD	mean	SD	mean	SD
ALB (g/L)	47.50	0.71	45.33	3.51	43.50	2.12
ALP (U/L)	97.00*	0.00	341.33	208.80	145.00	63.64
ALT (U/L)	76.50	6.36	53.67	12.50	66.00	11.31
AMY (U/L)	187.00	38.18	203.00	41.80	282.00	89.10
TBIL (µmol/L)	7.00	0.00	7.33	0.58	7.00	0.00
BUN (mmol/L)	8.60	0.28	9.57	0.68	8.20	0.71
P (mmol/L)	1.96	0.27	2.08	0.22	2.45	0.40
CRE (µmol/L)	125.00	5.66	75.00	9.00	80.00	21.21
GLU (mmol/L)	8.35	1.91	3.67	0.74	5.30	0.71
Na^+ (mmol/L)	161.00	5.66	156.33	6.51	159.50	2.12
K^+ (mmol/L)	4.00	1.27	4.20	2.69	5.15	1.34
TP (g/L)	58.00	2.83	61.00	9.54	57.50	0.71
GLOB (g/L)	10.50	3.54	15.67	10.07	14.00	2.83

** ALP value for males is taken from only 1 animal*

KEY: *ALB=albumin, ALP=alkaline phosphatase, ALT=alanine aminotransferase, AMY=amylase, TBIL=total bilirubin, BUN=urea nitrogen, CRE=creatinine, GLU=glucose, TP=total protein, GLOB=globulin, SD=Standard deviation*

3.2. HEMATOLOGY

The mean hematological values in Table 2 show variations between genders and between adults and sub-adults. The values for the male that was euthanized is excluded from these results.

Table 2. Hematology of eastern grey kangaroos from two populations in NSW during winter and spring 2014.

Leukocytes	Male (n=2)		Female (n=1)		Sub-adult male (n=2)	
	mean	SD	mean	SD	mean	SD
Neutrophils/100	55.83	6.83	41.00	0.00	48.50	13.44
Lymphocyte/100	27.00	9.90	35.66	0.00	33.00	13.20
Monocyte/100	7.33	0.47	9.66	0.00	7.16	2.12
Eosinophils/100	5.16	3.54	8.66	0.00	7.33	2.36
Basophils/100	4.66	0.00	5.00	0.00	4.00	0.47

The adult female had the highest lymphocyte count (35.66%). It was slightly higher than the lymphocyte counts for sub-adults (33.00%) and higher than the adult males (27.00%). Adult males had the highest neutrophil count (55.83%), whereas the lowest neutrophil count was recorded for the adult female (41.00%). A greater number of neutrophils than lymphocytes were observed for all animals in this study. The adult female had a neutrophil to lymphocyte ratio (NLR) of 1.15. Sub-adult males had more neutrophils with a NLR of 1.47. Adult males had the highest NLR of 2.07. The monocyte count for the adult female (9.66%) was higher than both adult males (7.33%) and sub-adults (7.16%). The adult female had a higher eosinophil count (8.66%) than the adult males (5.16%) and was slightly higher than the sub-adult males (7.33%). Sub-adult eastern grey kangaroos appear to have a slightly lower basophil count (4.00%) than adults of either gender (♂4.66%; ♀5.00%).

3.3. ANTIMICROBIAL ASSAY

Serum diluted to 25% was chosen for analysis because the bacterial colonies were clearer and easier to count accurately, compared to the colonies on plates with serum diluted to 50%. Test plates (containing serum) generally had far fewer CFUs than control plates containing heated serum or saline (Figure 2). However the trend was not observed in all plates, for example *Pseudomonas aeruginosa* control plates generally had fewer CFUs than test plates. Plates with serum had the fewest CFUs after 20 and 30 min of incubation.

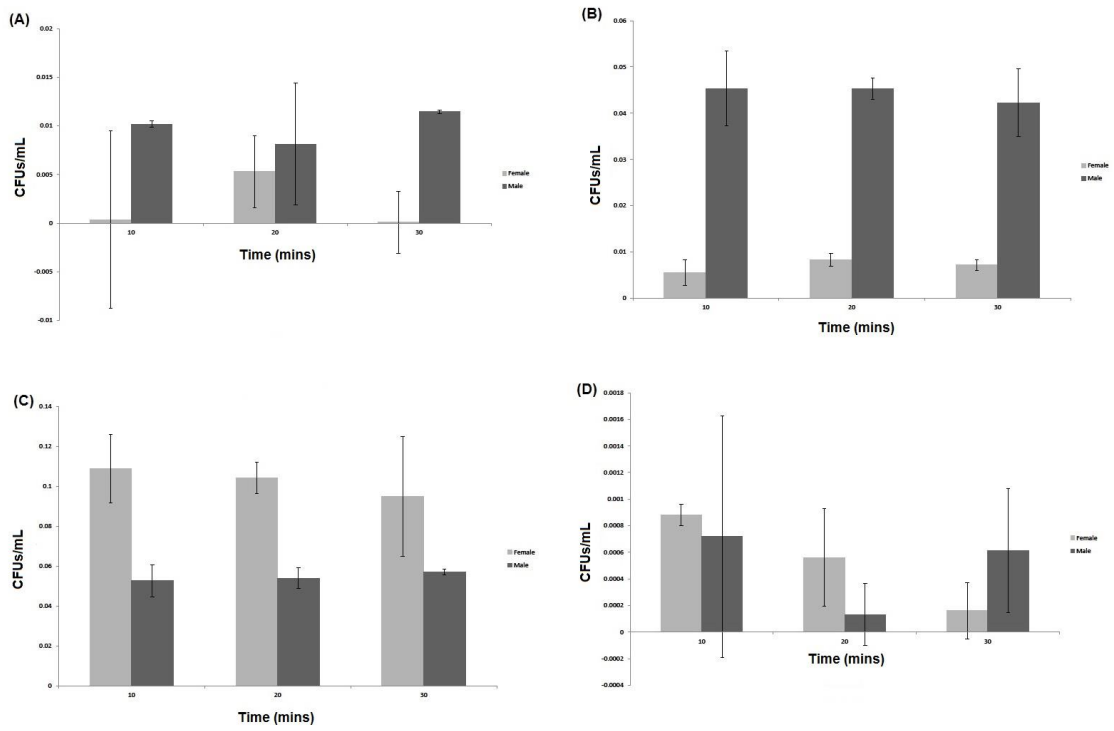

Fig. 2. Kinetics of antibacterial activity of 25% diluted serum from *Macropus giganteus* against **(A)** *Escherichia coli*, **(B)** *Staphylococcus aureus*, **(C)** *Pseudomonas aeruginosa* and **(D)** *Klebsiella pneumoniae.*

Test plates with *Klebsiella pneumoniae* had fewer CFUs than the controls. The number of CFUs increased at 10 minutes incubation, and the declined at 20 minutes incubation for all test plates, however the number of CFUs continued to decline after 30 minutes incubation for female test plates, but increased for male test plates.

Tests plates with male serum and *Escherichia coli* had fewer CFUs at 0 and 30 min; however they had larger numbers of CFUs than most control plates at 10 min and greater CFUs than some control plates at 20 min. The greatest CFUs were observed for the male test plate at 30 min incubation, and the fewest CFUs were observed at 20 min. Female results showed a clearer pattern. The female test plate and controls showed a sharp increase in CFUs at 20 min incubation followed by a sharp decline at 30 min.

Male test plates with *Staphylococcus aureus* had the fewest CFUs at 30 min incubation, with fewer CFUs than all controls at this length of incubation. Male test plates also had most CFUs at 0 min incubation. Female test plates had fewer CFUs at 10 min incubation and greatest CFUs at 20 min.

No evidence for adequate antibacterial response to *Pseudomonas aeruginosa* was observed. Test plates produced more CFUs than one or both of the controls on all occasions.

3.4. Acute Phase Protein Assay

3.4.1. Haptoglobin

The assay results indicate Hp is present in eastern grey kangaroos. Concentrations of Hp were determined from the Hp standard curve for both kangaroos (Table 3). Hp levels were higher for the euthanized kangaroo (6.96 mg/mL) compared to the healthy kangaroo (2.03 mg/mL).

Table 3. Mean concentration of haptoglobin in healthy and unhealthy (euthanized) eastern grey kangaroo serum.

Serum Specimen	Concentration of Unknown Sample (mg/mL)
Eastern Grey Kangaroo (unhealthy/euthanized)	6.96 (±0.10)
Eastern Grey Kangaroo (healthy)	2.03 (±0.12)
Rabbit	1.06 (±0.16)
Sheep	0.61 (±0.02)

3.4.2. SERUM AMYLOID A

Concentrations of SAA protein were determined from the standard curve for all kangaroos (Table 4). It was higher for adult females (6.13 µg/mL) than it was for healthy and unhealthy (euthanized) adult males (healthy=4.8 µg/mL / euthanized=4.27 µg/mL) and sub adult males (3.3 µg/mL). The SAA protein concentrations of all kangaroos were within the normal reference ranges specified by the SAA kit (<8 µg/). The horse serum showed a concentration of 32.67 µg/mL, which is notably higher than the normal reference range for horses (Jacobsen and Andersen, 2007).

Table 4. Mean concentration of serum amyloid A in adult male (healthy and unhealthy/euthanized), adult female, and sub-adult male eastern grey kangaroo serum.

Serum Specimen	Concentration of Unknown Sample (µg/mL)
Adult male (healthy) (n=2)	4.80 (±0.94)
Adult male (unhealthy/euthanized) (n=1)	4.27 (±2.27)
Adult female (n=3)	6.13 (±4.37)
Sub-adult male (n=2)	3.30 (±1.74)
Horse	32.67(±10.93)

4. DISCUSSION

The results of this research provide valuable information about the health parameters and disease susceptibility of free-ranging eastern grey kangaroos. Differences relating to age and gender were detected in both the hematological and blood chemistry values. Similarly gender, age and season have been determined to influence hematology and blood chemistry in other marsupials (Baker and Gemmell, 1999; Barnett et al., 1979; Haynes and Skidmore, 1991; Presidente, 1978). Some of the hematological and blood chemistry values in this study

were consistent with those found in other macropod species such as brush-tailed rock wallabies (*Petrogale penicillata*) and tammar wallabies (*Macropus eugenii*) (Barnes et al., 2008; Deane et al., 1997).

4.1. BLOOD CHEMISTRY

Levels of alkaline phosphatase were higher in female eastern grey kangaroos than in sub-adults, and both had higher levels than adult males. All females were lactating and had pouch young and two also had young at foot. Alkaline phosphatase has been found to dramatically increase during gestation in humans (Okesina et al., 1995). Similarly, pregnant bobcats (*Felis rufus*) reportedly have significantly higher alkaline phosphatase levels than males (Fuller et al., 1985). Alkaline phosphatase levels in female kangaroos may also fluctuate due to reproductive state. Alkaline phosphatase is involved in bone growth and calcification (Takenaka et al., 1988), therefore younger growing animals are likely to have higher alkaline phosphatase levels. For example, older eastern quolls (*Dasyurus viverrinus*) have been found to have approximately half the level of alkaline phosphatase as younger individuals (Stannard et al., 2013). Higher alkaline phosphatase values in younger animals has also been documented in tammar wallabies (McKenzie et al., 2002) and brush-tailed rock wallabies (Barnes et al., 2008), however the levels for both these species (tammar wallaby: 520-2357 U/L, brush-tailed rock wallaby: 405-1412 U/L) were substantially higher than the values found in the eastern grey kangaroos. Blood samples were collected from some animals in winter and others in spring which may have contributed to the differences in alkaline phosphatase levels between individuals. Studies have found that tammar wallabies and brush-tailed rock wallabies have higher alkaline phosphatase levels in summer than other seasons (Barnes et al., 2008; McKenzie et al., 2002).

Alanine aminotransferase was found to be markedly higher in males than females. Elevated levels of alanine aminotransferase in macropods may indicate issues with liver function (McKenzie et al., 2004). Overall, values are comparable with those recorded in the tammar wallaby (43-53 U/L) (McKenzie et al., 2002).

Adult males were found to have higher creatinine and glucose levels than females and sub-adults. Creatinine levels relate to muscle mass, therefore the higher levels of creatinine in males is most likely a reflection of their larger body size (Stirrat, 2003). It is possible that the adult males' body mass may have been understated as their large size made it difficult to weigh them accurately. It is likely that the male kangaroos actually weighed slightly more than the data suggests. Reduced creatinine levels may indicate poor nutrition (Domingo-Roura et al., 2001; Hall et al., 2007; Trumble et al., 2006). Higher creatinine levels in adult males have also been observed in brush-tailed rock wallabies, tammar wallabies and Tasmanian devils (*Sarcophilus harrisii*) (Barnes et al., 2008; Deane et al., 1997; Peck et al., 2015), however glucose was found to be lower in adults than sub-adults in all of these species (Barnes et al., 2008; Deane et al., 1997; Peck et al., 2015). Increased glucose levels are associated with increased stress (Barnett et al., 1979; Fuller et al., 1985; Stannard et al., 2013; Wells et al., 2000).

Potassium levels were higher in sub-adults than in males and females. Levels of potassium observed in sub-adults are comparable with the levels found in southern hairy-nosed wombats (*Lasiorhinus latifrons*) (5.7 mmol/L) (Gaughwin and Judson, 1980). Gaughwin and Judson (1980) suggest that high potassium values observed in wombats may be due to an adaptation to minimize water loss. Globulin levels were lowest in adult males compared with females and sub-adults. Globulin values that are higher in adults than sub-adults have been observed for brush-tailed rock wallabies (Barnes et al., 2008) and

Tasmanian devils (Stannard et al., 2016) which are not consistent with the higher levels in sub-adults found in this study. The levels of globulin observed were low compared to published values for other macropod species (Ruykys et al., 2012; Stirrat, 2003; Vaughan et al., 2009). All other values were similar to those published for other macropod species.

Stirrat (2003) suggested that macropod blood chemistry values are influenced by the availability and nutritional value of vegetation which varies between seasons. Lower levels of protein, urea nitrogen and albumin levels have been observed in agile wallabies (*Macropus agilis*) during spring compared to autumn (Stirrat, 2003). It is likely that seasonal variations may have impacted the values in the current study as samples were collected in both winter and spring.

4.2. HEMATOLOGY

The results of this study found that lymphocytes were higher in sub-adult eastern grey kangaroos than in adult males that are consistent with lymphocyte decrease associated with age in tammar wallabies (McKenzie et al., 2002). The adult female kangaroo which was lactating, had the highest lymphocyte value which compares with the increased lymphocyte values observed in brush-tailed rock wallabies during a time of high lactation demand (Barnes et al., 2008). Neutrophils were higher in adult males than both females and sub-adults. Conversely, tammar wallabies have higher neutrophil values at 2-3 years old, with the values of younger juveniles being similar to adults, and with values only dropping at around 3 years of age (McKenzie et al., 2002). The neutrophil to lymphocyte (N:L) ratio was 2.04 for adult males, 1.17 for adult females, and 1.45 sub-adult males.

Adult male and subadult monocyte values were fairly consistent, however the monocyte values of tammar wallabies have been shown to be lower at ages 0-1 and 3 years

old, peaking at around 2 years old and increasing again as an adult (McKenzie et al., 2002). The adult female had a notably higher eosinophil count than the males, and was lactating as this kangaroo had one pouch young, and one young at foot. The higher eosinophil value was in contrast with values observed in brush-tailed rock wallabies where eosinophil counts were notably lower during a time of high lactation demand (Barnes et al., 2008). A greater number of female eastern grey kangaroos in varying states of reproduction would be required to determine if eosinophil counts are significantly affected by lactation. Sub-adults were found to have higher eosinophil values than adult males and differs to that reported for the brush-tailed rock wallaby (Barnes et al., 2008), however the captive tammar wallaby has been found to have the highest eosinophil values at 2-3 years old, and slighter lower values at 0-2 years old and as adults (McKenzie et al., 2002). Barnes, Goldizen and Coleman (2008) suggest that higher eosinophil values may be related to exposure to parasites which would be greater in older animals. Higher eosinophil values in older individuals were not observed in eastern grey kangaroos, however the parasite loads for these animals is unknown. Higher density populations have a higher rate of contact between individuals which results in an increased endoparasite transmission rate (Takemoto et al., 2005). Eastern grey kangaroos were extremely abundant at both study sites. It is likely that the kangaroos at both study sites had high endoparasite burdens due to the high number of individuals at each site.

Overall variations in hematological values were observed between genders. No significant differences in hematological values have previously been reported between genders for this species. Both hematological and blood chemistry values have been found to differ among captive tammar wallabies of different stages of maturity and between seasons (McKenzie et al., 2002; Young and Deane, 2006), and likewise differences between brush-tailed rock wallabies of different ages and genders (Barnes et al., 2008).

4.3. *ANTIMICROBIAL ASSAY*

Staphylococcus aureus, and *Pseudomonas aeruginosa* infections have been reported in some macropod species, *Pseudomonas aeruginosa* has also been reported in koalas (*Phascolarctos cinereus*), and *Klebsiella pneumoniae* infection has been reported in common brushtail possums (*Trichosurus vulpecula*) (Edwards et al., 2012; Ladds, 2009; Osawa et al., 1992). These bacteria may also be present in eastern grey kangaroos.

Results indicate that eastern grey kangaroos have some level of antibacterial response towards the bacteria tested. Antibacterial responses to the Gram-negative bacteria *Klebsiella pneumonia* and *Pseudomonas aeruginosa* were generally less effective in females than males, and is consistent with the suggestion that Gram-negative bacteria are more abundant in marsupials that are in estrus, gestating, or carrying pouch young (Edwards et al., 2012). Numbers of Gram-positive bacteria have been found to be higher in female common brushtail possums during anestrus, estrus, gestation, or whilst carrying pouch young (Edwards et al., 2012). If these bacteria are present in eastern grey kangaroos, then it is likely there will be differences in the numbers of bacteria in males and females, and that bacterial levels will differ among females during different reproductive states. In contrast, the antibacterial response to the Gram-positive bacteria *Staphylococcus aureus* was notably higher in males, which is not consistent with the suggestion that numbers of Gram-positive bacteria are higher in females. The abundance of Gram-negative bacteria *Escherichia coli* was also higher for males than females. Edwards (2012) suggests that certain strains of bacteria in the pouch of marsupials may be excluded or favored by maternal mechanisms and that a selective response towards Gram-negative bacteria occurs in preparation for parturition.

The decreased bacterial growth after longer incubation indicates that antibacterial effects increased over time. Conversely, Merchant et al. (2013; 2003) found a decrease in

antibacterial effects over time in Komodo dragons (*Varanus komodoensis*) and American alligators (*Alligator mississippiensis*), however these studies used longer incubation times of 3h, 6h and 12h. It is unknown whether the antibacterial effects of eastern grey kangaroo serum would continue to increase or decrease after 30 min as this was the longest incubation time used in this study.

4.4. HAPTOGLOBIN

In this study, the concentration of Hp protein in eastern grey kangaroos was successfully measured, and confirms its presence in eastern grey kangaroo serum. The serum from the euthanized kangaroo showed a marked elevation in Hp protein concentration. Acute phase proteins are stimulated by infection, trauma and inflammation (Cray et al., 2009). It is likely that the euthanized kangaroo may have had an inflammatory condition, however the normal blood sera Hp range for kangaroos is not known because there is no sufficient data available on acute phase proteins in marsupials.

Hp studies on ungulates using 151 serum samples from wild species in the Bovidae, Cervidae, and Equidae families have shown that Hp levels were similar to those of related domestic animals (Stefaniak et al., 1997). Infections and injuries contributed to increased Hp levels in the sera, and suggested the potential and usefulness of observing the Hp levels as part of a routine examination for the diagnostics of inflammation in wild species (Stefaniak et al., 1997). Hp concentration in the grey short-tailed opossum (*Monodelphis domestica*) has been shown to increase almost six-fold after the injection of lipopolysaccharide (Richardson et al., 1998), which suggest that, measuring Hp levels may also be a valuable method of identifying diseases and other inflammatory disorders in marsupials including kangaroos.

4.5. *SERUM AMYLOID A ASSAY*

All kangaroos in this study had SAA protein concentrations within the normal reference ranges specified by the SAA kit (<8 μg/). SAA protein concentration was highest in adult females, followed by adult males, and subadults had the lowest SAA concentration.

There was no difference in the protein concentration of SAA between the euthanized adult male and the healthy adult males. SAA is an indicator of acute inflammation however the slower reacting Hp indicates chronic inflammation (Alsemgeest et al., 1994; Horadagoda et al., 1999). The injuries of the euthanized kangaroo were more consistent with a chronic condition, that may explain why Hp protein levels were elevated, but SAA levels were not. The results from this study are preliminary findings that demonstrate the use of this kit for measuring levels of SAA protein in marsupial serum. The horse serum in this study showed a concentration of 32.67 μg/mL, which is slightly higher than the normal reference ranges but lower than the SAA level of horses with an inflammatory condition (Jacobsen and Andersen, 2007).

5. CONCLUSIONS

This paper contributes additional data to the scarcity of information on the hematology and blood chemistry values of free-ranging eastern grey kangaroos, and reports the ability of their serum to inhibit bacterial growth. It also reports that marsupials, like eutherians, are capable of producing APPs that are triggered during an infection or trauma event.

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